
BIOSENSORS BASED ON NANOPOROUS TiO_2 FILMS

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Abstract

The use of nanoporous TiO_2 films as solid substrates for protein immobilisation has been investigated. Such films are of interest due to their high surface area, optically transparency, electrochemical activity and ease of fabrication. These films moreover allow detailed spectroscopic study of protein / electrode electron transfer processes.

It is found that protein immobilisation on such films may be readily achieved from aqueous solutions at 4 °C with a high binding stability and no detectable protein denaturation. The nanoporous structure of the film greatly enhances the active surface area available for protein binding (by a factor of up to 850 for an 8- μm -thick film). It is demonstrated that the redox state of proteins such as immobilised cytochrome-c (Cyt-c) and haemoglobin (Hb) may be modulated by the application of an electrical bias potential to the TiO_2 film, without the addition of electron transfer mediators. The binding of Cyt-c and Hb on the TiO_2 films is investigated as a function of film thickness, protein concentration, protein surface charge, pH and ionic strength.

Furthermore, it is shown that protein / electrode electron transfer may be initiated by UV bandgap excitation of the TiO_2 electrode. Both photooxidation and photoreduction of the immobilised proteins can be achieved. By employing pulsed UV laser excitation, the interfacial electron transfer kinetics can be monitored by transient optical spectroscopy, providing a novel probe of protein / electrode electron transfer kinetics.

Additionally, optical and electrochemical biosensors have been developed based on protein immobilisation on the TiO_2 films. The fluorescence yield of immobilised fluorophore labeled maltose binding protein (MBP) may be used to monitor specifically maltose and β -cyclodextrin concentrations. The potential use of immobilised *Hb* for the optical detection of dissolved carbon monoxide and nitric oxide in aqueous solutions is

also demonstrated. Finally, the electrical conductivity of the films in combination with the catalytic properties of the immobilised Hb have been used for the development of an amperometric nitric oxide biosensor.

It is concluded that nanoporous TiO₂ films provide a solid surface to immobilise proteins in a stable and functional way. These films may be useful both for basic studies of protein / electrode interactions and for the development of novel bioanalytical devices, such as biosensors, employing both optical and electrochemical signal transduction methodologies.

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Abbreviations

CB	Conduction Band
CE	Counter Electrode
CO	Carbon monoxide
Cyt- <i>c</i>	Iron Cytochrome <i>c</i>
Cyt- <i>c</i> /TiO ₂	Cyt- <i>c</i> adsorbed on a TiO ₂ film
Cyt- <i>c</i> /ZnO	Cyt- <i>c</i> adsorbed on a ZnO film
CV	Cyclic Voltammetry
ΔOD	Changes in Optical Density
Hb	Hemoglobin
Hb/TiO ₂	Hemoglobin adsorbed on a TiO ₂ film
IEP	Isoelectric Point
IANBD	N,N'-dimethyl-N-(iodoacetyl) -N'-(7-nitrobenz-2-oxa-1,3- diazol-4-yl) ethylenediamine
K _D	Binding constant
MBP	Maltose Binding Protein (wild type)
MTA	Microsecond Transient Absorption
MW	Molecular Weight
NO	Nitric Oxide
OD	Optical Density
RE	Reference Electrode
RT	Room Temperature
S337C-NBD	IANBD labeled MBP
SEM	Scanning Electron Micrograph
TiO ₂	Titanium dioxide
VB	Valence Band
UV-Vis	Ultraviolet-Visible
WE	Working Electrode
ZnO	Zinc Oxide

CHAPTER 1

INTRODUCTION

1.1 Motivation

There is currently considerable interest in the immobilisation of biomolecules upon electrodes and other surfaces. The reasons for such a drive in research are numerous and diverse spanning from the most fundamental studies of protein function and structure (Hirst et al., 1998 and Jones et al., 2000) to the development of bioanalytical devices such as biosensors (Price et al., 2001 and Rusling et al., 2000, Cass et al., 1984, Shinohara et al., 1991 and Georganopoulou et al., 2000). My thesis studies developed from initial experiments, which had indicated that nanoporous TiO_2 films may provide a novel substrate for protein immobilisation. The aim of my doctoral studies was to investigate in depth this immobilisation strategy, focusing both upon its potential use for the development of novel biosensors, and for studies of protein/electrode electron transfer kinetics.

1.2 Biosensors

A biosensor is an analytical device composed of a biological recognition element directly interfaced to a signal transducer, which together relate the concentration of an analyte to a measurable response (Hall et al., 1991 and 1998). Biosensors are increasingly becoming practical and useful, if not essential, tools in medicine, environmental monitoring, food quality control and research and therefore their development is an area of research, which continues to create great interest. In principle, they can be tailored to match individual analytical demands for almost any target

molecule or compound that interacts specifically with a biological system (Scouten et al., 1995). A biosensor makes use of a biological molecule that is immobilised to a transducer to detect specifically a target analyte. The binding or conversion (if the analyte is a substrate) event leads to an optical, mass, thermal or electrochemical (conductometric, amperometric and potentiometric) response that is related to the analyte concentration in the sample (Ingersoll et al., 1997, Scheller et al., 1992 and Guilblaut et al., 1984) (see Figure 1.1).

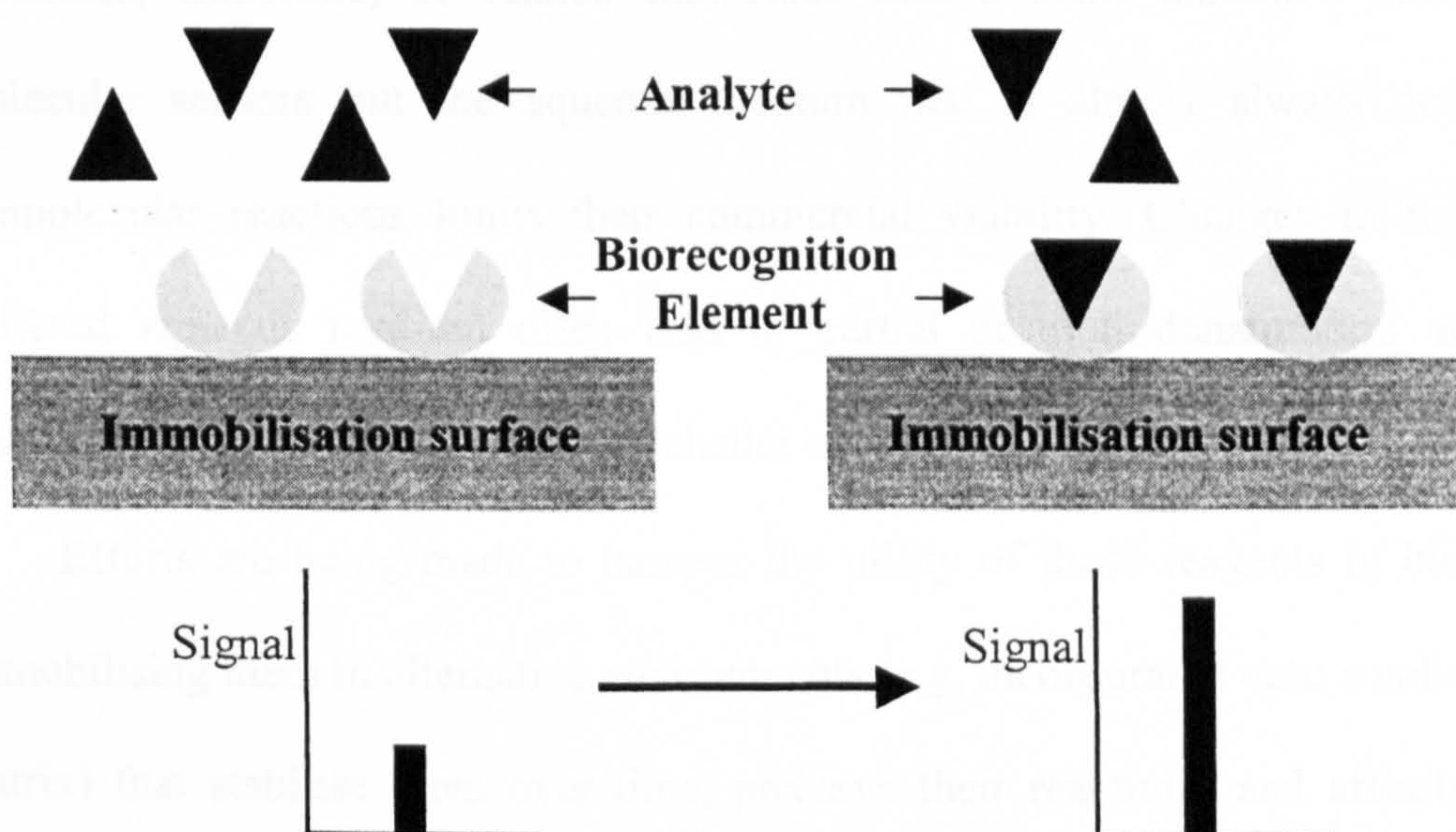


Figure 1.1: Diagram of a biosensor.

Of course, there are many steps associated with the actual development of a real biosensor, such as the choice of an appropriate biorecognition element to selectively recognise the analyte, the selection of a biocompatible surface and immobilisation strategy for the effective and strong binding of the biomolecule on the surface and the

selection of a detection scheme. Issues that remain unresolved, however, include assay reversibility and the use of multistep procedures that require addition of components other than the analyte of interest.

Proteins or enzymes are often chosen as molecular recognition molecules, which are the key elements in the fabrication of optical and electrochemical sensors (Janata et al., 1998, Wang et al., 1999, Hall et al., 1997, Popescu et al., 1997, Scouten et al., 1995, Guilbault et al., 1984). Protein electrodes represent the oldest group of biosensors and are being increasingly used for clinical testing of metabolites such as glucose, lactate, urea and creatine. The specificity and fast reaction rates of proteins for binding of substrates, inhibitors, or related molecules makes them attractive candidates for molecular sensors but the aqueous medium that is almost always necessary for biomolecular reactions limits their commercial viability. Changes in the preferred buffered aqueous medium often lead to partial or total denaturation and loss of reactivity (Hermanson et al., 1992, Scheller et al., 1992).

Efforts are being made to harness the utility of these reagents in biosensors by immobilising them in alternative environments (e.g. incorporated onto a solid surface or matrix) that stabilise them over time, preserve their reactivity and affinity, does not block signal transmission and the biomolecule can be reset/reused. Optimum biosensor design requires maximum retention of biomolecular reactivity as well as efficient, cost-effective signal transduction.

1.2.1 Electrochemical biosensors

Electrochemical and optical signal transduction are the most popular signalling methods for the development of biosensor devices in recent years. An amperometric (electrochemical) biosensor makes use of a protein (e.g. oxidoreductase) that is immobilised in proximity to a transducer (electrode) to detect an analyte, and ultimately transduces the chemical signal produced by the interaction into a measurable response (electrical current) (Janata et al., 1998, Popescu et al., 1997). The current produced is linearly proportional to the concentration of the analyte. The major concern in the development of amperometric biosensors has been directed towards the fast and reversible electron transfer between the active site of the immobilised protein and the electrode surface. Although these devices are the most commonly reported class of biosensors, they tend to have a small dynamic range due to saturation kinetics of the protein. However, the main advantages of these devices is the low cost (disposable electrodes are often used with this technique) and the high degree of reproducibility of the measurements.

1.2.2 Optical biosensors

The basis of an optical biosensor occurs when the binding of a target molecule is accompanied by a unique change in the optical spectroscopic characteristics of the immobilised biomolecule (optical signal transduction), usually absorption or fluorescence (Blyth et al., 1995, Wang et al., 1999). Therefore the solid surface required for the development of optical biosensors should be optically transparent, porous

enough to allow diffusion of analyte molecules, to provide a high biomolecule loading per unit area and to preserve the structure and activity of the immobilised protein. The main advantages of these biosensors are the speed and reproducibility of the measurements. The main drawback of optical measurements is the high cost of the apparatus and that these instruments are generally larger than is practical for on-site measurements.

1.3 Protein immobilisation

A critical step for successful signal transduction and development of biosensors is effective protein/enzyme immobilisation on a suitable solid surface. The major advantages are successful electrical contact between the protein's redox center and the electrode's interface and that the protein molecules are immobilised in a stable and functional way to allow studies of many analyte solutions. Other advantages are close control of the reaction medium and conditions, prevention of chemical and bacterial degradation, cost-effective reusability of the protein, and enhanced biomolecular stability. However, proteins often fail to retain their native stabilities and reactivities upon immobilisation, a flaw that results in low stabilities or altered functional responses of biosensors incorporating them.

Various conventional methods have been described for enzyme or protein immobilisation (Wang et al., 1994, Foulds et al., 1988, Luong et al., 1993) (see Table 1.1). For optimum biostability and reaction efficiency, the preferred host matrix appears to be one that isolates the biomolecules, protecting them from self-aggregation and

microbial attack, while at the same time providing essentially the same local aqueous microenvironment as in biological media.

Protein engineering and particularly site directed mutagenesis is a procedure applied on most of the methods, described on table 1, in order to improve the binding of the biomolecules on the surfaces (Hill et al., 1987, Hellinga et al 1998a and 1998b). The advantages are oriented protein immobilisation on the surface, improvement of the stability of the immobilised protein and production of more stable biocatalysts. The disadvantages are that mutants are used instead of native proteins and the characteristic chemical and physical properties of the proteins are more or less changed.

METHOD	ADVANTAGES	DISADVANTAGES
Adsorption on insoluble matrices (e.g. by Van der Waals forces, ionic binding or hydrophobic forces)	Simple, mild conditions, less disruptive to enzyme protein	Enzyme linkages are highly dependent on pH, temperature and solvent; insensitive
Covalent binding onto a membrane or insoluble supports	Stable enzyme-support complex, leakage of the biomolecules is very unlikely, ideal for mass production and commercialisation	Complicated and time consuming; possibility of activity losses due to the reaction involving groups essential for the biological activity (can be minimised by immobilisation in the presence of the substrate or inhibitor of the enzyme)
Crosslinking by a multifunctional reagent (such as glutaraldehyde bis-isocyanate derivatives or bis-diazobenzidine)	Simple procedure, strong chemical binding of the biomolecules; widely used in stabilising physically adsorbed proteins that are covalently bound to a support	Difficult to control the reaction; requires a large amount of the enzyme, the protein layer has a gelatinous nature (lack of rigidity), relatively low enzyme activity
Entrapment and Encapsulation in polymeric matrices	Universal for any enzyme, mild procedure	Large diffusional barriers, loss of enzyme activity by leakage, possible denaturation of the protein molecules as a result of free radicals, difficult to get electrical connectivity

Table 1.1: Immobilisation procedures for proteins

1.4 Bioelectrochemistry of redox proteins

Bioelectrochemistry is now a well-established discipline for investigation of the redox chemistry of enzymes (Willner et al., 2000, Hill et al., 1996, Bowden et al., 1997, Taniguchi et al., 1997, Dong et al., 1997, Lotzberger et al., 1997, Katz et al., 1997, Harmer et al., 1985). Similarly the immobilisation of metal or semiconductor electrodes with redox proteins is central to recent advances in bioanalytical and biocatalysis technologies. Electrochemical activation of redox proteins is of great importance in the development of amperometric biosensors. Electrocatalytic reduction or oxidation of the protein allows the quantitative determination of the analyte by the transduced amperometric output of the electrode. Therefore, amperometric biosensor devices require the integration of redox-proteins with electrode surfaces in an organised configuration exhibiting electrical contact.

The protein/electrode complex is also an attractive concept from the perspectives of biology (Willit et al., 1987). The protein/electrode complex is a useful analogue of protein/protein complexes, which are the basis of many biological electron transfer reactions. In contrast to bioelectrochemical studies of protein redox reactions at electrode surfaces, for solubilised redox proteins the method of choice for kinetic studies of biological electron transfer is transient optical spectroscopy. Such studies are facilitated by the high optical densities readily achieved in a 1 cm optical cuvette. The low optical densities arising from mono-layer coverage of an electrode surface, combined with the fact that most electrodes are not optically transparent, has generally precluded optical studies of protein/electrode electron transfer reactions.

The electrical contact between the protein's redox center and the electrode's interface is required (see figure 1.2) but is usually prohibited due to the insulation of the active site (heme) by the protein matrix.

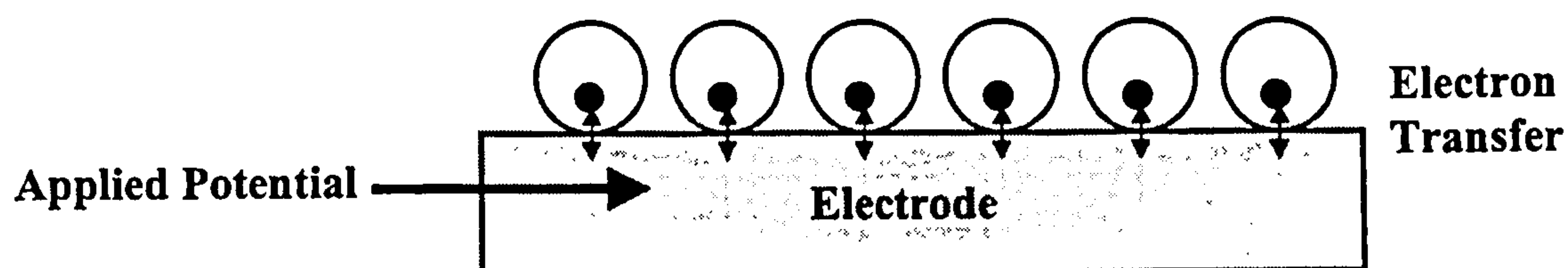


Figure 1.2: Ideal protein monolayer coverage of an electrode surface. The protein molecules are positioned in such a way to allow their hemes to come into direct contact with the electrode surface so that fast interfacial electron transfer can take place.

The electron transfer theory (Marcus et al., 1985) implies that the electron transfer rate between a donor and an acceptor site is given by the following equation (Bendall et al., 1996).

$$k_{et} \propto e^{-\beta(d-d_0)} e^{\frac{-(\Delta G^0 + \lambda)^2}{4RT\lambda}}$$

Where ΔG^0 is the change in free energy associated with electron transfer and the generation of the redox products; λ is the reorganisation energy associated; d_0 is the van-der-Waals distance; d is the actual distance separating the donor and the acceptor; β is the electronic coupling coefficient. Usually, the protein assembly separates the redox-site from the electrode surface and practically insulates the active center from electrical contact with electrode interfaces.

Electron carrier proteins, and particularly cytochrome *c* (Cyt-*c*), have been shown to undergo strong adsorption at a number of electrode surfaces (Szucs et al., 1995), including mercury (Scheller et al., 1975), gold (Cooper et al., 1993, Willner et al.,

1999), silver (Cotton et al., 1980), tin oxide (Brown et al., 1996), indium oxide (Tarlov et al., 1991 and Taniguchi et al., 1997) and ruthenium dioxide electrodes (Hill et al., 1985).

Several strategies have been employed in order to optimise the transfer of electrons between the protein and the electrode's surface (Katz et al., 1997). Diffusional electron mediators such as ferrocenes, ferricyanide, N, N'- bipyridinium salts, and quinone derivatives were applied as charge transporters that connect the active redox center and the electrode's surface. These mediators operate by a diffusional route where penetration into the protein matrix permits short electron transfer distances with respect to the active site and simultaneous diffusion of the electron carrier from the protein facilitates electrical contact with the electrode's surface.

A further means to establish electrical communication of redox proteins with electrodes includes the surface modification of the electrode with promoter molecules (promote the electrochemistry) (Hill et al., 1993) that bind and align the protein to a configuration that facilitates electron transfer. This method is usually applicable to relatively low molecular weight proteins, i.e. cytochrome *c* (Qu et al., 1994). Promoters are distinguished from mediators since while the latter take part in electron transfer mechanisms with the formation of reduced and/or oxidised intermediates, promoters simply aid the electron transfer without taking part in electron transfer.

Another method reported to yield electrical contact was chemical modification of redox proteins by redox-tethered components (e.g. ferrocene units covalently linked to the redox protein) (Degani et al., 1989). By this method, electron transfer distances are shortened and the redox groups associated with the protein act as electron relays to transport the charge to or from the protein's active site.

Finally, immobilisation of redox enzymes in redox polymer matrices (e.g. ferrocene modified polymers or Os(II)-polypyridine substituted polymers) was used as an additional means to electrically communicate the redox center with electrode surface (Degani et al., 1989). At these modified electrodes rapid electron transfer can take place. However, conflicting results showed that the commercially available samples of Cyt-c, without further purification, usually only yield unstable, ill-defined voltammograms. Alternatively at promoter modified electrodes well-developed cyclic voltammograms have been obtained from a sample as received. Adsorption on the metals has been found to be irreversible and some denaturation of the protein occurs upon adsorption, except in the case of tin oxide where it maintains its native state (Collinson et al., 1992). Immobilisation of the protein on solid electrodes, such as tin oxide, offers a way of investigating the electron transfer of a donor/acceptor interface without the presence of diffusion processes. Such interactions also have important applications for analytical and clinical biochemistry.

These approaches have already achieved some success e.g. the development of the ExacTech glucose electrode (Hill et al., 1996). For this biosensor ferrocenes acted as excellent mediators between glucose oxidase and an electrode and the current produced was linearly related, up to an appropriate level, to the amount of glucose in solution. Therefore it was successfully used to measure blood glucose levels of people who suffered from diabetes.

However, the details of the protein/electrode interactions and in particular the rates of protein/electrode interfacial electron transfer remain only poorly characterised both experimentally and theoretically. This is even for the case for direct electrochemistry between the protein and the electrode surface. This complicates the design and optimisation of the protein/electrode interface.

1.5 Sol-gel encapsulation

One approach to develop surfaces that are optically transparent with high monolayer coverage that can be used for the development of optical biosensors is the use of sol-gel methods to encapsulate the protein of interest. Traditional inorganic glasses are not generally considered to be a suitable host matrix for immobilisation of biological molecules. Consequently, stabilisation of proteins and enzymes within inorganic silica glasses is very unusual. The breakthrough in immobilisation methods has been made possible by the sol-gel method of synthesising inorganic solids.

There is currently considerable interest in the use of sol-gel methods for the encapsulation of biological macromolecules, aimed primarily at the development of optical bioanalytical devices (Dave et al., 1994 and 1997, Blyth et al., 1995, Ellerby et al., 1992, Dunn et al., 1998, Lev et al., 1995, Lan et al., 1999) but also at providing a controlled environment for studies of biomolecular function (Bhatia et al., 2000, Shen et al., 1997, Miller et al., 1996, Ji et al., 1998, Chen et al., 1998). These studies have focused upon the encapsulation of a range of proteins in a growing covalent gel network rather than being chemically bound to an inorganic matrix. Studies have focused upon the encapsulation of a range of proteins in silica glasses, where the porosity of such glasses allows the diffusion of small analyte molecules to the immobilised protein (Dunn et al., 1998, Ellerby et al., 1998, Dave et al., 1997, Blyth et al., 1995).

The sol-gels have been reported to be chemically, thermally and structurally stable, and proteins immobilised by this procedure retain their functional characteristics to a large extent. In addition the porosity of such glasses allows the diffusion of small analyte molecules to the immobilised protein. The high biomolecule loading per unit area and the optical transparency of the glass makes this approach particularly suitable for optical signal transduction methodologies (Blyth et al., 1995, Ellerby et al., 1992,

Chung et al., 1995, Glezer et al., 1993). The encapsulation of biological molecules in sol-gel materials requires stringent control over gelation processes because the biomolecules tend to denature in environments with high alcohol concentration and/or extreme pH values as well as elevated temperatures (Lapanje et al., 1978). This has motivated the development of modified versions of the gelation processes, which employ milder environmental conditions to minimise denaturation of the encapsulated biomolecules (Dunn et al., 1998, Liu et al., 1999).

Another problem with this method is that the properties of the gel continue to change as long as solvent remains in its pores, since crosslinking is still taking place in the solid amorphous phase. Eventually, the strength of the gel increases and the pores become smaller. The control of crosslinking to produce a sol-gel with reproducible and controllable pore sizes must be achieved before the procedure can be considered for practical applications in biosensor construction.

1.6 Motivation and Aims of this PhD study

1.6.1 Alternative immobilisation procedure: TiO₂ Films

In this thesis an alternative approach to protein immobilisation allowing both optical and electrochemical studies of the immobilised proteins is considered: immobilisation upon preformed nanoporous titanium dioxide (TiO₂) films. TiO₂ is a

metal oxide and a wide band gap (~ 3.2 eV) semiconductor and therefore optically transparent and non-scattering for wavelengths ~ 350 nm. It is environmentally benign, with for example TiO_2 particles being widely used as additives in toothpaste and white paint. Large quantities of nanoporous TiO_2 films can be readily prepared by low cost screen printing technologies (O'Regan et al., 1991). Such films typically comprise a rigid, porous network of 10-20 nm TiO_2 anatase nanoparticles, as illustrated in the scanning electron micrograph shown in figure 1.3. The pore sizes are 5-20 nm, sufficiently large for proteins to diffuse throughout the porous structure. The surface area of such films is greatly enhanced over flat electrode surfaces (up to 1000 fold for an 8 μm thick film).

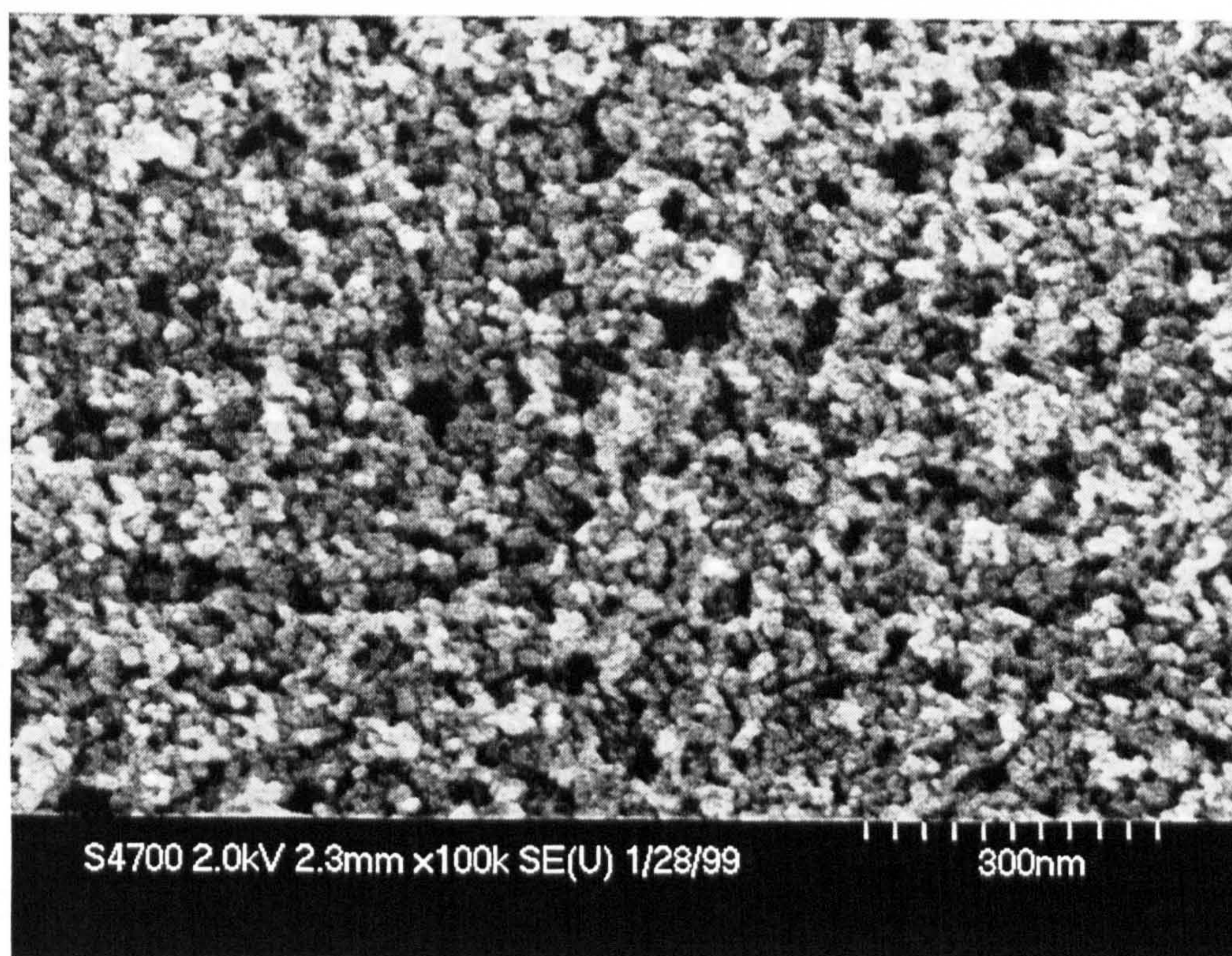


Figure 1.3: SEM picture of an 8 μm thick nanocrystalline TiO_2 film.

In addition to their optical transparency and high surface area, these films exhibit good stability, and electrochemical activity at potentials above the conduction band edge. A network of interconnected mesoscopic TiO_2 particles allow for electronic conduction to take place. In recent years, there is a considerable growth and commercial interest in studies of the technological potential application of these films. They are currently being widely studied for applications ranging from photoelectrochemical solar cells to lithium batteries, light-emitting diodes and electrochromic windows (O'Regan et al., 1991, Cummins, et al., 2000, Sotomayor et al., 2000). Further studies for example investigated the electrocatalytic activity of vitamin B_{12} adsorbed to such nanoporous TiO_2 films for electrosynthesis (Mbindyo et al., 1998) .

The high surface area, conductivity and optical transparency of the nanoporous TiO_2 films suggest they may also provide a suitable surface for both bioanalytical applications (e.g. biosensors) and for fundamental studies of protein/electrode and protein/protein electron transfer reactions.

This is the first reported study describing the use of the nanoporous TiO_2 films for protein immobilisation. Specifically this thesis addresses the following issues:

- The use of the nanoporous TiO_2 films as an alternative substrate for protein immobilisation
- Characterisation of the protein binding and investigation of the parameters that control it.
- The ability to apply direct electrochemistry on the immobilised proteins without the use of electron transfer mediators or promoters.

- Characterising the protein/electrode (TiO_2) electron transfer kinetics using transient absorption spectroscopy.
- The ability to use the nanoporous TiO_2 films for the development of optical and electrochemical biosensors.

CHAPTER 2

EXPERIMENTAL METHODS

2.1 Sample preparation

This section gives a detailed description of the preparation of all samples studied in this thesis. The first part of this section outlines the preparation of nanoporous, nanocrystalline titanium dioxide films and the second part of this session deals with the preparation of the protein solutions. Subsequently, details of protein adsorption on nanoporous titanium dioxide films are outlined.

2.1.1 Preparation of nanocrystalline TiO₂ films

The preparation of nanocrystalline colloidal films can be best described in three simple steps:

1. Preparation of an aqueous solution of colloidal 15 nm size particles of TiO₂ (concentration 12.5% titanium dioxide by weight).
2. Deposition of the colloidal solution on a conducting glass substrate.
3. Sintering the deposited films at elevated temperatures to achieve electrical contact between the particles.

2.1.2 Preparation of TiO₂ colloidal paste

An aqueous suspension of colloidal TiO₂ particles was synthesised by employing a sol-gel reaction details of which are given in (Barbe et al., 1997). The titanium dioxide particles used in this thesis were synthesised in Dr. James Durrant's laboratory at Imperial College by Dr. Thierry Lutz. The TiO₂ paste comprised of 15 nm size particles and was 12.5% titanium dioxide by weight. The phase of the TiO₂ was determined as anatase. At all times the paste was stored in the fridge at 5°C prior to and after use.

2.1.3 Deposition of TiO₂ on glass substrates

The titanium dioxide paste was mixed with polyethylene glycol (PEG) mw 20,000. The amount of PEG added to the titanium dioxide paste corresponded to 50% of the weight of titanium dioxide itself in the paste. The carbowax provided a support structure for the film while it forms and improves the homogeneity of the film. In addition, it increases the porosity of the titanium dioxide film and consequently allows for more protein adsorption. The TiO₂/PEG mixture was left stirring very slowly for at least 5 hours at room temperature to ensure complete blending of the PEG and the titanium dioxide paste. The stirring must be very slow in order to avoid generation of bubbles which cause inhomogeneous films.

The titanium dioxide paste was deposited onto fluorine doped tin oxide coated conducting (TEC-15) glass slides purchased from Hartford Glass, Indiana which have a resistance of 15 Ω/sq. Prior to deposition, the surface of each 2.5 x 7.5-mm glass slide was cleaned with ethanol to ensure that it is dry and lint free. Great care was taken not

to damage the conducting surface while cleaning. The clean glass slide was then carefully placed onto a flat clean glass surface and both edges were fixed firmly with strips of Scotch Magic tapeTM while allowing a separation of 1 cm as shown in figure 2.1. The number of layers of tape used determined the thickness of the film. Typically, n layers of tape yielded $4n \text{ }\mu\text{m}$ thick films where n is the number of tape layers. Then a sufficient amount (0.2 ml) of TiO_2 paste was introduced onto one of the un-taped ends of the glass slide and was quickly and evenly spread down the length of the conducting glass slide using a glass rod or a Pasteur pipette. This if done correctly, gave a nice homogeneous film. This film making technique, commonly referred to as doctor blading, is shown in figure 2.1.

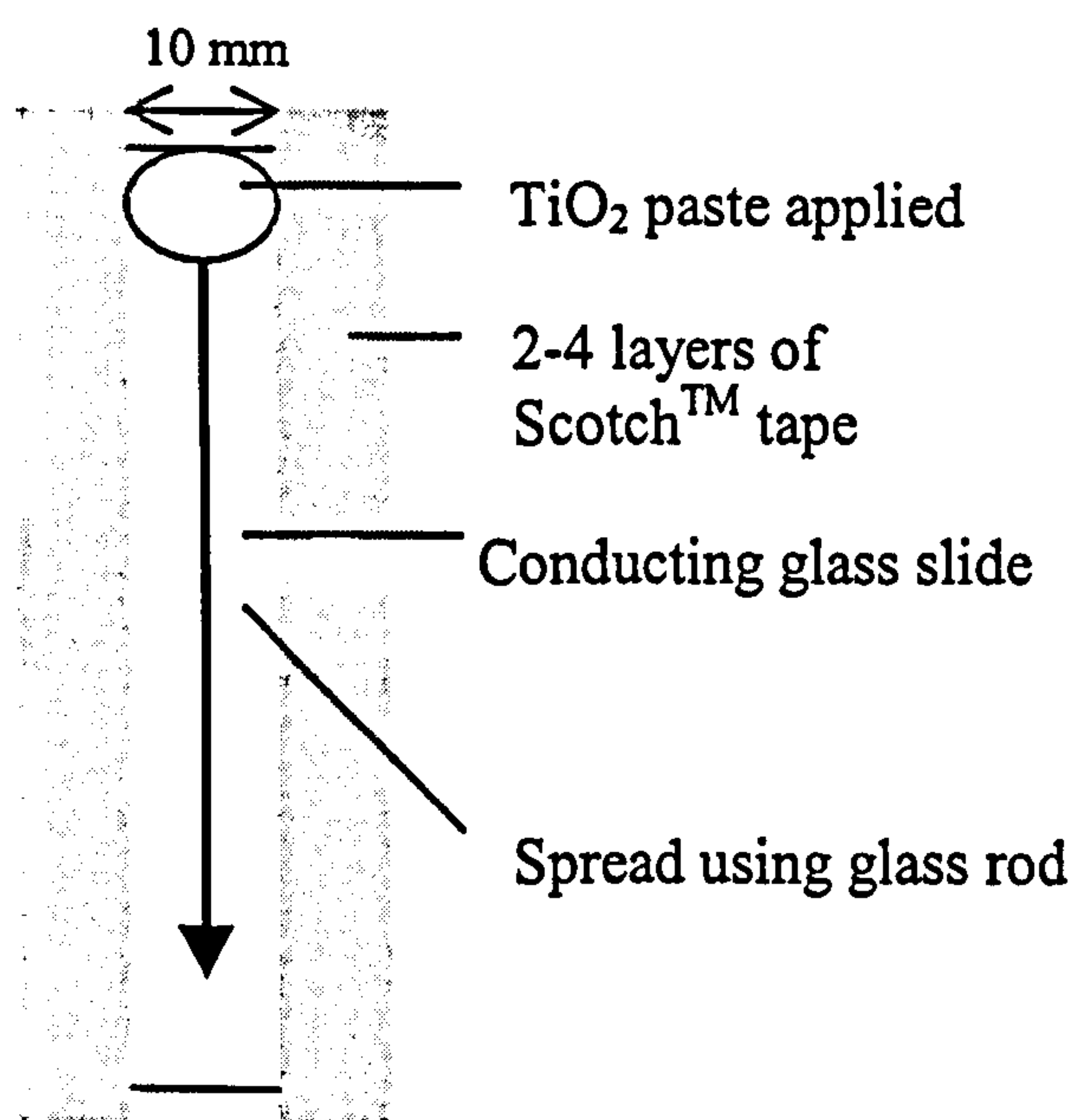


Figure 2.1: The 'doctor blade' method being employed in the preparation of nanocrystalline TiO_2 films at Imperial College. A conducting glass slide was taped down and a glass rod being used to spread the TiO_2 paste.

After the deposition of the paste the strips of tape were carefully removed and the film was placed in a clean dust-free environment for approximately 20-30 minutes to dry in air.

2.1.4 Film sintering

After this time the white TiO_2 paste spread on the conducting glass slide had become transparent and it was sufficiently dry to be sintered in a hot-air drying apparatus. The apparatus was designed to allow the film to dry in a hot air flow at a specific and constant temperature. Attempts to heat the film before it becomes transparent resulted the cracking of the film. The air-dried films were sintered at 450°C under airflow generated by an airgun for 25 minutes. Great care was taken not to exceeding sintering temperatures above 500°C as the conversion of the crystal phase TiO_2 particles from anatase to rutile may be accelerated above this temperature. The prolonged heating removed the PEG leaving only the nanocrystalline TiO_2 particles behind. The heat also sintered the nanocrystalline particles together providing good electrical contact throughout the film. Once the heating was finished, the films were allowed to cool gradually to room temperature under airflow. All the sintered films were then stored in a sealed plastic box under anhydrous conditions. The sintered film consists of nanometer sized particles and resembles a sponge like structure.

Accuracy in film preparation is vital because the crystalline nature of the film and the quality of the electrical contact between the particles are influenced by the preparation conditions.

2.1.5 Preparation of protein solutions

Horse-heart ferricytochrome *c*, type IV, (Cyt-*c*), bovine-blood Haemoglobin (Hb), horse-heart Myoglobin (Mb) and Acetylated ferricytochrome *c* (*Ac* Cyt-*c*) were obtained from Sigma Chemical Co. as lyophilised powders and were used without any further purification. Maltose binding protein (S337C-NBD) was prepared following a published procedure (Gilardi et al., 1994). The aqueous immobilisation solutions comprised the protein of interest and 10 mM NaH₂PO₄ or KH₂PO₄ buffer solutions (obtained from BDH chemical Co.), with the pH adjusted to a value of 7 or 8 using NaOH. All aqueous solutions were prepared in distilled, de-ionised water of resistance $R = 10 \text{ M}\Omega \text{ cm}$.

2.1.6 Immobilisation of proteins on TiO₂ films

The immobilisation of proteins on the TiO₂ films is a relatively simple procedure. The nanocrystalline TiO₂ glass slides were broken up into 10-mm wide strips. Then a piece of TiO₂ film was carefully washed with ethanol, placed in the hot air drying apparatus and heated at 450°C for 10 minutes. The reason for this is to remove any adsorbed species on the TiO₂ film surface. After the 10 minutes, the hot air gun was switched off but the blowing was allowed to continue until the temperature had dropped to 25°C. At this point the film was taken out with tweezers and was dipped in 10 mM NaH₂PO₄, pH 7 buffer for a few seconds. This was done in order to cool the film down before it comes in direct contact with the protein solution because it might denature partially or completely the protein molecules.

Protein immobilisation was achieved by the immersion of the 1-cm² TiO₂ film in 4 ml of aqueous protein solutions at 4°C for 1-30 days depending upon protein and immobilisation conditions. Protein adsorption onto the TiO₂ films was monitored by recording the UV/visible absorption spectra of the immobilised films at room temperature on a Shimadzu UV-1601 spectrophotometer. Contributions to the spectra from scatter and absorption by the TiO₂ film alone were subtracted by the use of protein free reference films. Prior to all spectroscopic measurements, films were removed from the immobilisation solution and rinsed in buffer solution to remove non-immobilised protein. After the immobilisation of proteins had reached saturation the resulting films were immersed in protein free buffer solution for up to one month and any protein desorption was monitored over time.

2.1.7 Zinc oxide films

The nanocrystalline ZnO films were made in Professor Michael Grätzel's laboratory in EPFL by Dr. Bryan O'Regan. The aqueous paste of colloidal ZnO particles was synthesised by employing a sol-gel reaction details of which are given in (Hilgendorff et al., 1998). The ZnO paste comprised of 15 nm size particles and was 20% ZnO by weight ethyl cellulose. The ZnO films were optically transparent and 4 μm thick. Cyt-*c* immobilisation was achieved by the immersion of 1 cm^2 ZnO films in protein solution at 4 °C for 1-7 days.

2.2 UV/Visible absorption spectroscopy

The absolute absorption spectrum of a protein may be shown as a plot of the light absorbed (extinction) by the protein against wavelength. Such a plot for a coloured protein will have one or more absorption maxima in the visible region of the spectrum (400-700 nm). All absorption spectrophotometers consist of a source of radiation, a sample cell and a detector. In this thesis, UV-visible spectroscopy was performed using a Shimadzu 1601 UV/Vis recording spectrophotometer. The spectral resolution of the spectrometer was 2 nm and spectra were collected with a sampling interval of 1 nm. UV-visible spectroscopy was used to monitor the adsorption and the desorption of different proteins on the optically transparent nanoporous TiO_2 films.

2.3 Spectrofluorimetry

Fluorescence is the phenomenon whereby a molecule, after absorbing radiation, emits radiation of a longer wavelength. Thus a compound may absorb radiation in the ultraviolet region and emit visible light. A spectrofluorimeter consists of a continuous spectrum source (Xenon arc), a monochromator for irradiating the sample with any chosen wavelength, a second monochromator which enables the determination of the fluorescent spectrum of the sample and a detector which is usually a sensitive photocell (photomultiplier). All fluorescence measurements were made on a Perkin-Elmer LS-50 spectrofluorimeter at room temperature. The TiO_2 films immobilised with protein were placed at a fixed angle (see figure 2.2) in a 3-ml fluorescence cuvette, filled with 3 ml of 10 mM KH_2PO_4 of pH 7.

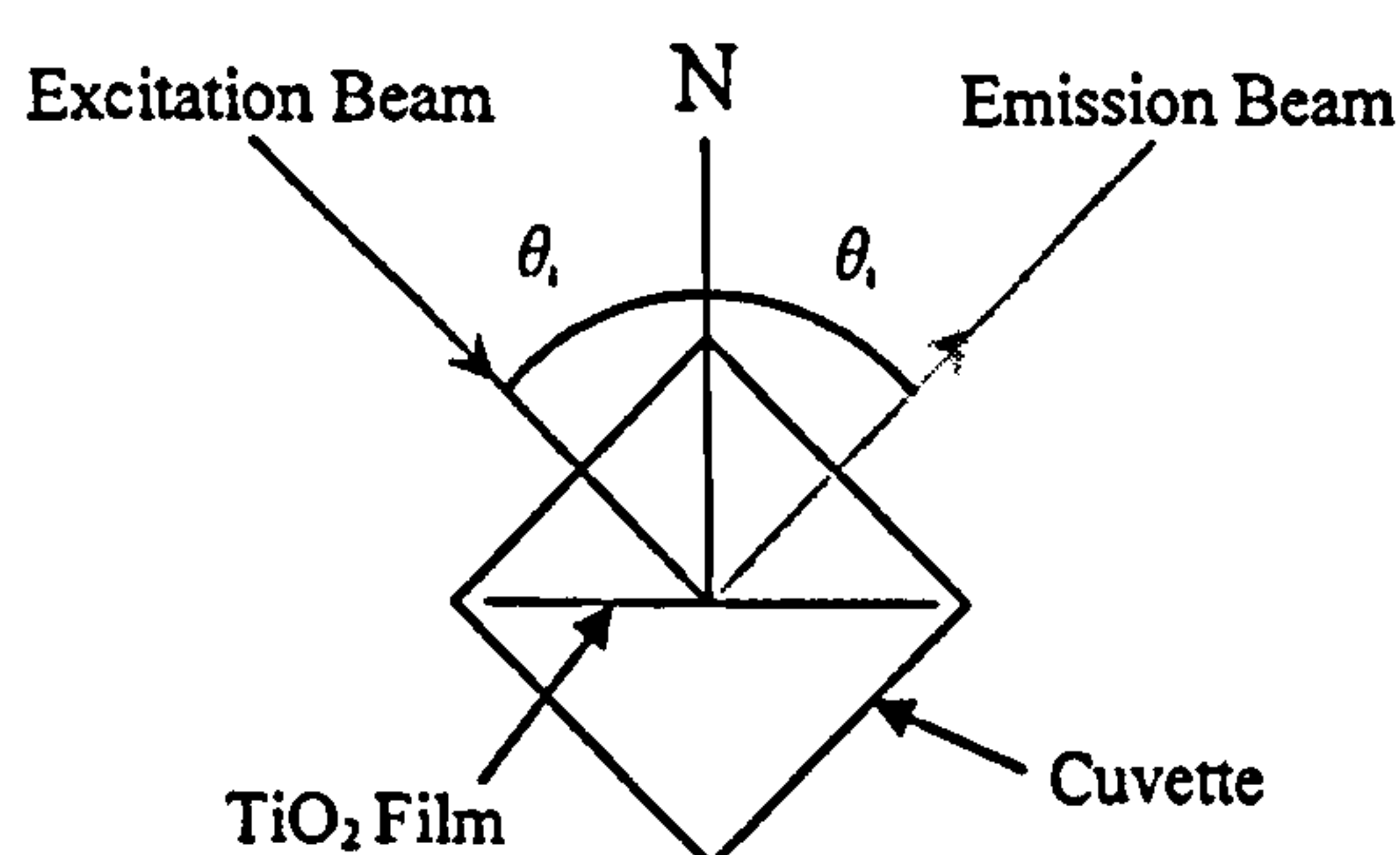


Figure 2.2: This illustration shows the position of the TiO_2 film in the cuvette.

2.4 Spectroelectrochemistry

A cell constructed with an optically transparent electrode (OTE) can be used in a spectrophotometer to follow spectroscopically the course of electrochemical reactions. Upon the application of a potential the electroactive species will be reduced/oxidised and equilibrium is established. This technique is used in my thesis to study changes on the absorption spectrum of the immobilised with protein TiO_2 film while it is subjected to a variety of electrical potentials.

2.4.1 The Electrochemical cell

A three electrode cell (see figure 2.3) was employed for all spectroelectrochemical and electrochemical studies. Three electrodes are required for an electrochemical investigation: the working electrode (WE) which is under investigation, a counter electrode (CE) which takes up the current, and a reference electrode (RE). The potential of the WE is measured with respect to that of the RE. It is important that the ohmic potential drop between the WE and the RE is as small as possible. The potential of the WE is controlled by a potentiostat.

The electrochemical cell was used to investigate the influence of an external applied voltage upon the redox state of protein adsorbed nanocrystalline TiO_2 films.

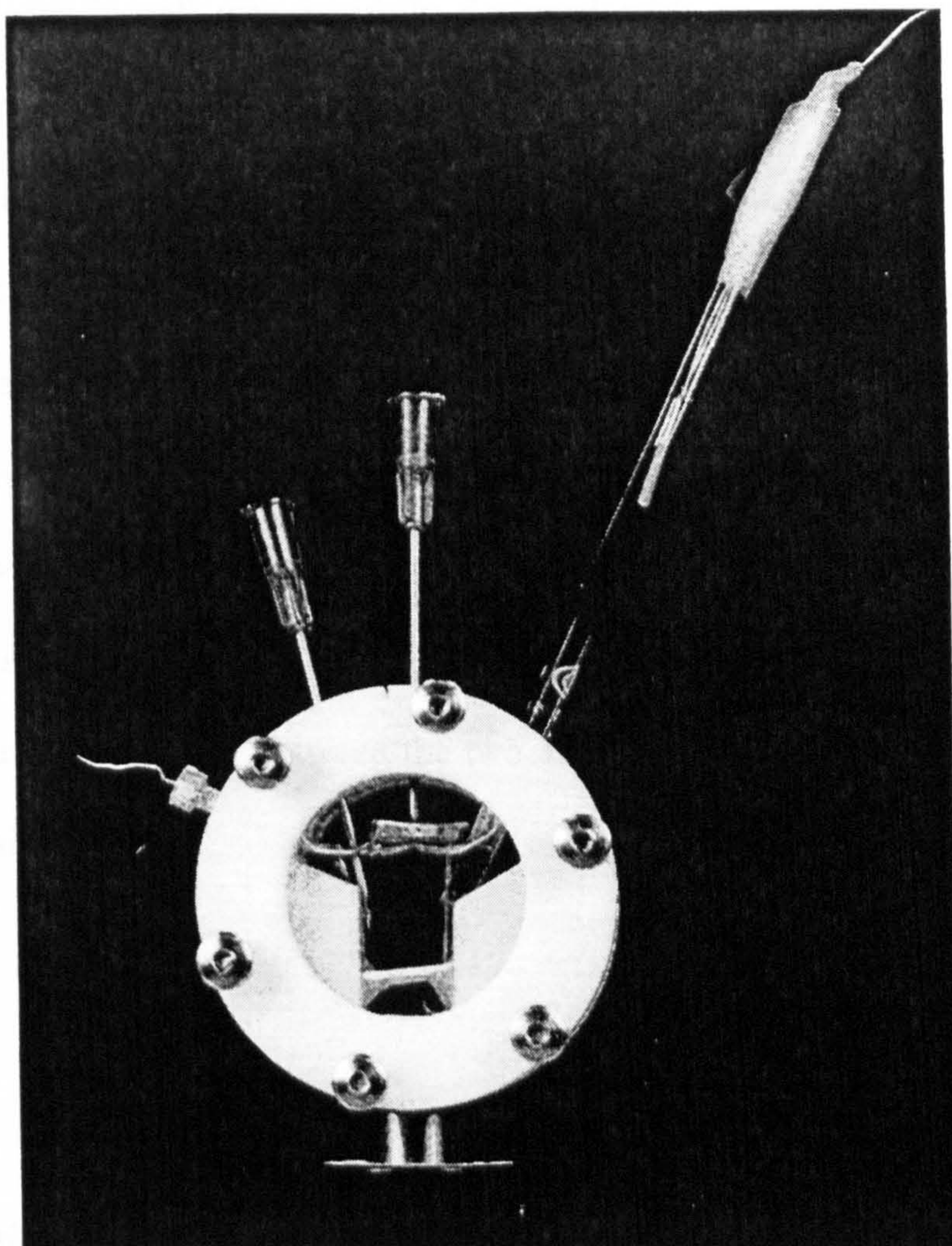


Figure 2.3: Picture of the three electrode electrochemical cell. The middle electrode in the working electrode, which is the protein adsorbed titanium dioxide film, clipped with some platinum foil and attached to an iron needle. The platinum foil allows the electrical connection between the iron needle and the conducting glass. The electrode on the left-hand side is the counter electrode, which is a platinum flag (platinum wire attached to some platinum mesh). The electrode on the right hand site is the Ag/AgCl electrode (saturated KCl).

The three-electrode cell was carefully designed with an optical path to allow steady state absorption experiments to be performed. Therefore two circular quartz glass windows of 4 cm diameter were obtained from Sarna Optical Glass Company and were used as the walls of the electrochemical cell. The protein adsorbed nanocrystalline titanium dioxide electrode formed the working electrode. A platinum flag was used as the counter electrode. The reference electrode was chosen to be a standard Ag/AgCl electrode which was purchased from BDH Chemicals and all potentials quoted are with reference to this standard. The RE electrode was positioned very close to the WE to minimise the solution resistance between the two and hence to control the potential as accurately as possible.

2.4.2 Preparation of the working electrode

A 10 mm wide strip of nanocrystalline TiO_2 on conducting glass immobilised with protein, was scratched with a diamond scribe on the conducting layer to one side, just underneath the sensitised area. This isolated a section of the glass, reduced any effect the exposed conducting tin oxide layer may have had on the signals that were recorded during the experiments and prevented any short circuit. This film was used as the working electrode of the electrochemical cell.

2.4.3 The Electrolyte solution

An electrolyte is a substance that dissolves in a solvent to produce a conducting solution of ions. Sometimes the solution as a whole is termed electrolyte. Within electrolytes it is possible to have strong ones and weak ones depending on the degree of ionisation. A 10 mM NaH_2PO_4 buffer solution of pH 7 was the electrolyte used in all the spectroelectrochemical and electrochemical experiments, without the addition of redox mediators or promoters.

2.4.4 Spectroelectrochemistry on proteins immobilised on TiO_2 films

All Spectroelectrochemical measurements were conducted in the homebuilt 3 electrode electrochemical cell comprising the TiO_2 film as working electrode. The electrochemical cell was clamped in place in the Shimadzu spectrophotometer which was connected directly to a pentium PC running the spectrophotometer's own data collection software. The beam path of the spectrophotometer was narrowed so that it passed only through the area of the conducting glass covered by TiO_2 and immobilised by protein. Potential control was provided by an Autolab PGstat 12 computer controlled potentiostat. The potentiostat supplied positive and negative bias and measured very low photocurrents. The top of the spectrophotometer was covered to stop stray light interfering with the experiments. Background light interference proved to be minimal. The electrolyte solution used was 2.5-3.5 ml of 10 mM NaH_2PO_4 of pH 7, which was degassed with argon prior and during any optical / electrochemical measurements unless otherwise stated.

2.5 Electrochemical techniques

Electrochemistry can be defined generally as the study of chemical reactions aiming to produce electrical power or the use of electricity to affect chemical processes. Cyclic voltammetry (CV) and chronoamperometry (CA) were used in this thesis to characterise the nanoporous TiO_2 and ZnO films before and after protein immobilisation.

2.5.1 Cyclic voltammetry

CV is one of the most versatile and widely applicable electrochemical techniques for use when an initial qualitative assessment of the electrochemical behaviour of a system is required. Procedurally, it is relatively simple and the necessary equipment is ubiquitous in most electrochemical laboratories.

The kinetics of electrode processes are commonly studied by CV. The technique of voltammetry is essentially the monitoring of change of current with the variation of electrode potential. The electrode potential is varied linearly at a certain rate and the response of the system to the applied potential gives rise to changes in the current. When the potential approaches the formal potential of the species in solution, a current is observed due to the oxidation/reduction process. The process is plotted against the potential and the overall shape of the curve gives aspects of the kinetics of the electrode processes (figure 2.5). This method uses a triangular potential scan (see figure 2.4) i.e. as the potential applied to a solution is scanned first towards more positive potentials the

rate v_1 (and therefore current) increases until oxidation occurs. Having swept to the highest voltage E_2 , the potential comes back down at the same rate v_2 . Then the potential is reversed and scanned back to the starting potential E_1 giving the characteristically triangular potential-time function. On the reverse sweep the oxidised species, having been created during the forward sweep, is reduced by the opposite process to that in the forward sweep. This reduction gives a current in the opposite sense to that in the forward scan which peaks and then falls due to depletion of oxygen in the diffusion layer. This behaviour gives rise to the characteristic voltammogram of a reversible system in solution (see figure 2.5).

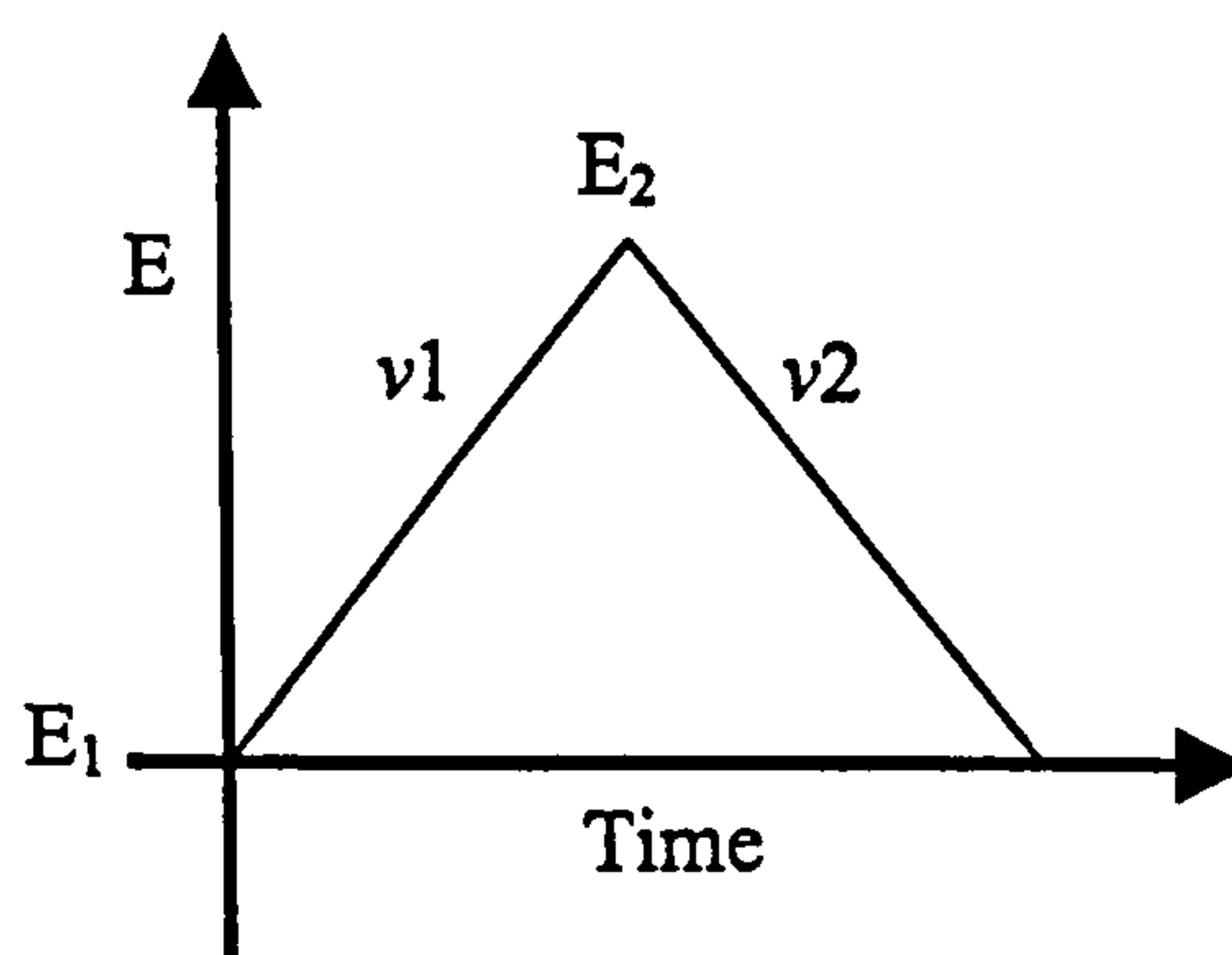


Figure 2.4: Typical voltage (E) changes during cyclic voltammetry.

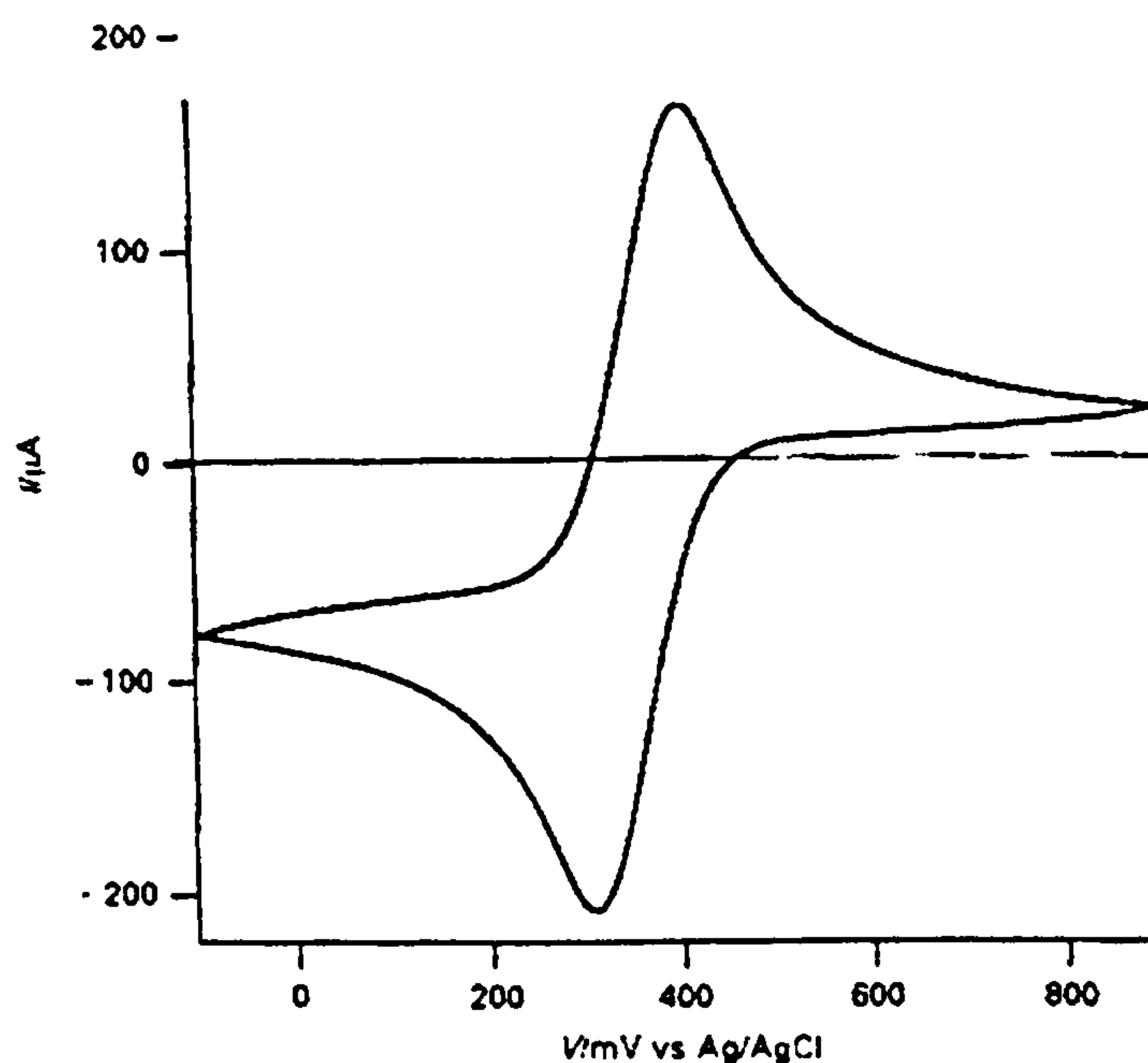


Figure 2.5: Characteristic Cyclic Voltammogram of a reversible system.

For a redox protein immobilised on an electrode, protein film voltammetry (PFV) can be used (Hirst et al., 1998). PFV differs from traditional protein voltammetry in that, rather than examining proteins in solution and relying on mass transport to bring them transiently to the electrode surface, PFV examines a film of protein molecules adsorbed directly onto the electrode surface. For example proteins have been spontaneously adsorbed on unmodified graphite electrodes in a redox-active state. Direct adsorption gives a protein (sub)monolayer which is tightly locked into the electrode potential. Having adsorbed the protein, the cyclic voltammetric response should consist of a pair of peaks corresponding to reduction and oxidation of the active site. As shown in figure 2.6, at sufficiently low scan rates this signal should be Nernstian, the peak separation should be zero and both peaks should be symmetrical

with equal half height widths (δ) of $0.304T/n$ mV where T is in Kelvin and n is the number of electrons involved in the process.

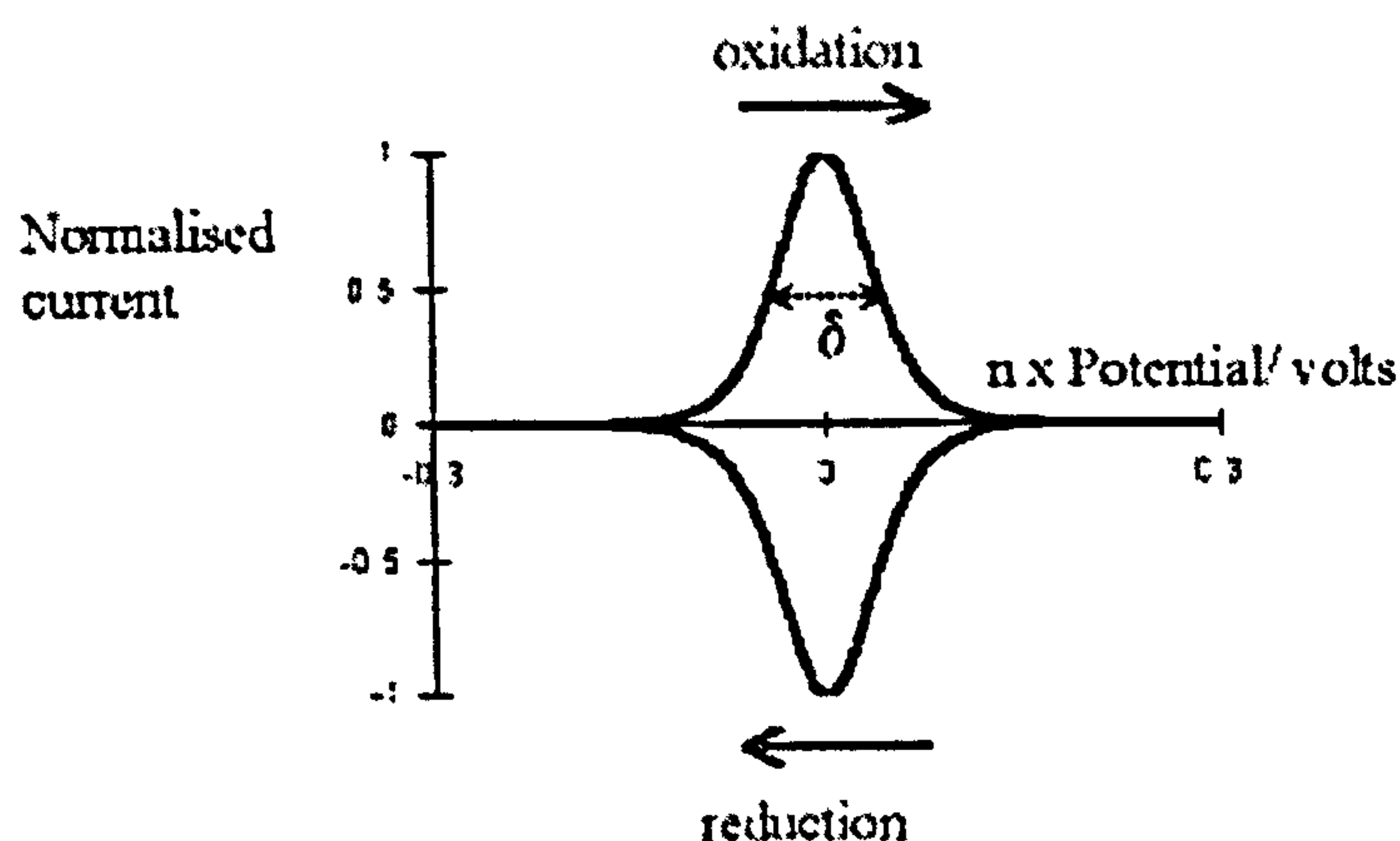


Figure 2.6: Ideal voltammogram for an adsorbed redox protein undergoing reversible and uncoupled interfacial electron transfer. The peaks are symmetrical and occur at the same potential (the reduction potential) in each direction.

Cyclic voltammetry on blank or immobilised with protein TiO_2 or ZnO films was carried out, using the 3 electrode cell and the Autolab PGSTAT10 computer controlled potentiostat, over the potential range -0.7 to $+0.1$ V vs the Ag/AgCl electrode at a scan rate of 0.1 V/s and at a step potential of 0.00488 V unless otherwise stated. The electrolyte solution used was 10 mM NaH_2PO_4 buffer solution of pH 7 unless otherwise stated. All experiments were carried out at room temperature.

2.5.2 Chronoamperometry

Chronoamperometry, in which a current versus time curve is measured after a step potential is applied to the electrode, is one of the most important techniques in electroanalytical chemistry. Chronoamperometry employs three electrodes in a steady solution. The potential difference between the working and the reference electrode starts out at 0 and no redox reaction can occur at the working electrode. The voltage then jumps immediately on WE and the potential difference between the WE and RE is great enough to induce a redox reaction at the WE. The redox reaction immediately depletes the oxidant in the vicinity of the electrode while increasing the reductant. The resultant density gradient leads to a flow of oxidant toward the electrode and reductant away from the electrode. The moving charge is the diffusion current. The current response to this sweep in potential is characterised by an immediate jump in current, which drops off as the oxidant is depleted. The diffusion current can then be related to the analyte concentration. The applied potential is constant for a given time and then drops back to the initial value. In this thesis, CA was used for the development of a nitric oxide biosensor based on the immobilisation of Hb on TiO₂ films.

2.6 Steady state photoreduction

Steady state photoreduction of proteins immobilised on the TiO_2 films was conducted by illumination of the protein/ TiO_2 films in 10 mM phosphate buffer solutions by the filtered output of a 150 Xe lamp. A 330 nm low pass filter was used and the resulting light intensity incident on the sample was 4.2 mW cm^{-2} . The experimental set-up used to carry these experiments is presented in figure 2.7.

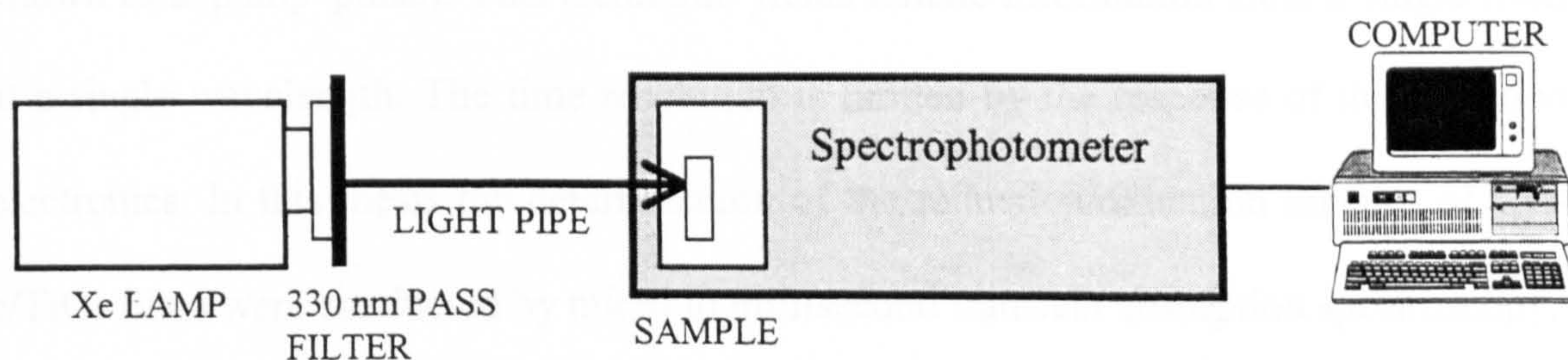


Figure 2.7: Experimental set-up for steady state photoreduction of protein/ TiO_2 films.

2.7 Transient absorption spectroscopy (Flash photolysis)

2.7.1 Principle

The continuously probed transient absorption spectroscopy, commonly termed flash photolysis, is a technique which involves monitoring the transmission of a continuous probe beam following the change in optical density of the sample, induced by the 'pump' pulse (The irradiation of the sample with a short intense pulse of light is known as a 'pump' pulse). This technique yields kinetic information from a single flash at a single wavelength. The time resolution is limited by the response of the detection electronics. In this thesis the determination of the reduction/oxidation kinetics of Cyt-c/TiO₂ films were conducted by micro to millisecond transient absorption spectroscopy.

2.7.2 The microsecond transient absorption (MTA) spectrometer

All microsecond transient absorption measurements were carried out by employing a nitrogen laser. The apparatus was designed and developed by Dr. James Durrant (Durrant et al., 1992). In this section a description of the set-up of the spectrometer is summarised. The optical layout of the microsecond transient absorption spectrometer is shown in figure 2.8.

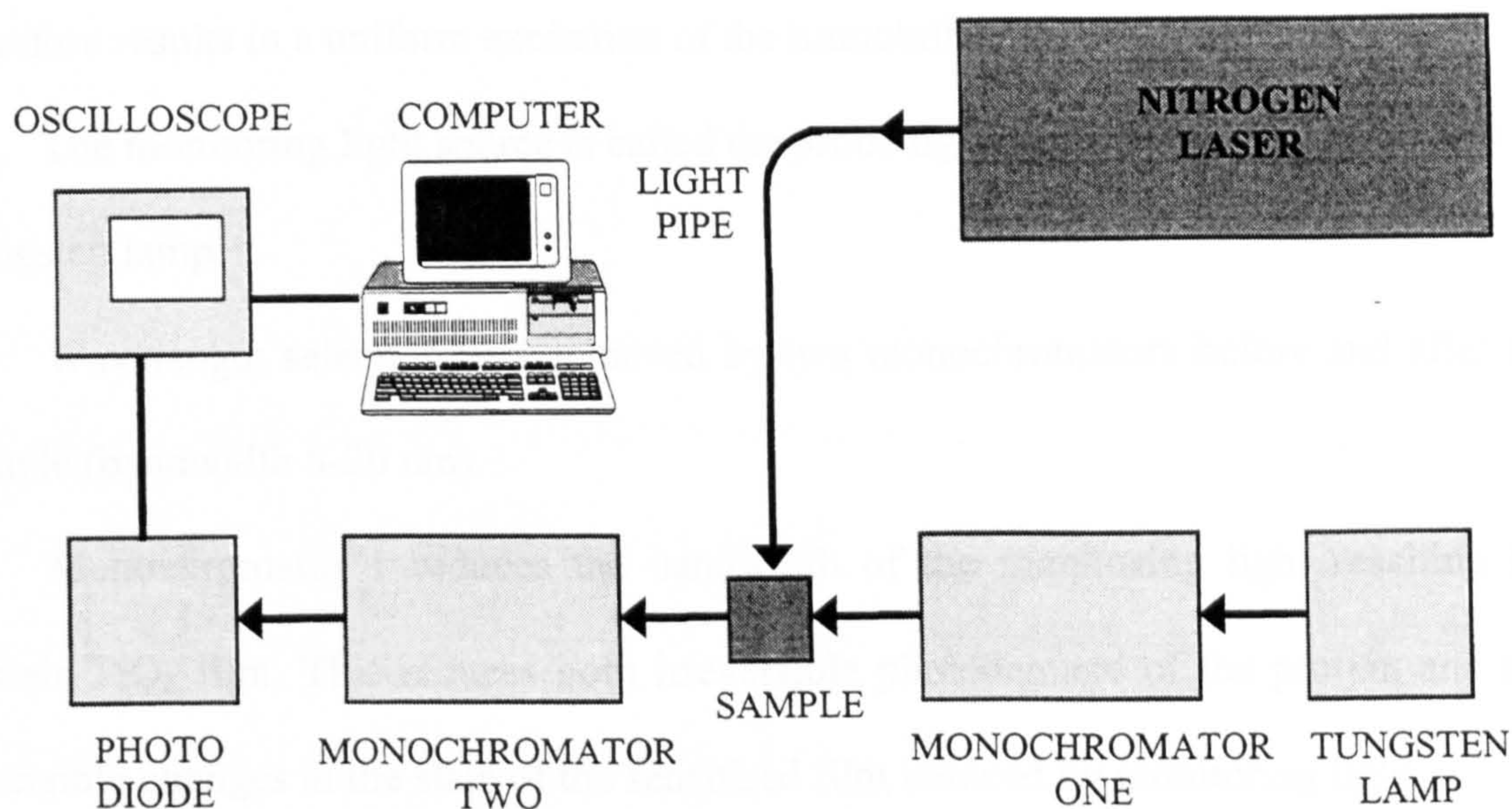


Figure 2.8: The optical layout of the microsecond to millisecond transient absorption spectrometer. A nitrogen laser is used as the excitation source. A liquid light pipe transmits the excitation pulse to the sample. A tungsten lamp is used as the probe source.

A PTI GL-3300 Nitrogen Laser was used as the excitation source. This laser yielded 600 ps laser pulses at a wavelength of 337 nm with a pulse energy of 0.3 mJ cm^{-2} at a repetition rate of 0.1 Hz. A light pipe was used to transmit the excitation pulse to the sample. The output beam from the nitrogen laser is potentially blinding, and this beam is therefore focused into a light guide in an enclosed volume immediately in front of the laser. The output from the light guide has a more

homogeneous cross-sectional shape than the output beam from the nitrogen laser and therefore results in a uniform excitation of the immobilised film.

The monitoring light source is called the probe light and was delivered by a 150 W Tungsten lamp.

Wavelength selection was achieved by two monochromators before and after the sample (bandwidth 8-20 nm).

Monochromator 1 reduces the bandwidth of the monitoring light reaching the protein/TiO₂ film. This reduces both irreversible photodamage of the protein and any reversible changes in the state of the sensitised film induced by monitoring light.

Monochromator 2 provides the bandwidth resolution of the system and reduces the intensity of fluorescence and laser light scatter reaching the photodiode by several orders of magnitude, depending upon the wavelength used.

Changes in optical density induced by the excitation pulses were monitored by a Si photodiode and custom-built amplification/filtering electronics. These electronics are crucial in determining the system's time resolution and high frequency signal to noise.

A Tektronics TDS220 oscilloscope digitises, averages and stores the amplified transient signal. A computer is linked to the oscilloscope, which allows the storage of data on hard and floppy discs, the use of a more efficient averaging routine, and access to advanced data analysis programs like Origin.

2.7.3 Transient absorption kinetics of protein/TiO₂ Films

Proteins were immobilised on TiO₂ films as described in section 2.1.6. The absorption spectrum of the protein/TiO₂ films was recorded prior to any laser excitation experiments. Then the protein/TiO₂ film was immersed in a thin, 1 ml, quartz cuvette containing 1 ml of 10 mM NaH₂PO₄ buffer solution of pH 7 or 3 mM potassium ferricyanide and was positioned in the laser set-up (see figure 2.7). The protein/TiO₂ film was excited with the delivered pump UV light, which has a wavelength of 337 nm. At this wavelength the immobilised protein does not absorb but there is strong bandgap absorption of the TiO₂ film. The photooxidation and photoreduction of the immobilised protein were measured by changes in the transient absorption spectra at two wavelengths, one where absorption changes of the protein occur when it is reduced and one where they don't, under laser flashes. At the end of the transient absorption experiments the absorption spectrum of the protein/TiO₂ film was recorded again. Control data were collected with TiO₂ films with no adsorbed protein.

2.7.4 Calculation of ΔOD

Transient absorption spectroscopy involves the detection of changes in the intensity of monitoring light transmitted through the sample. The excitation pulse causes these changes. The data collected by the oscilloscope is readily converted to units of ΔOD , in the limit of small absorption changes, by using the following equation:

$$\Delta OD(t) = \alpha \times \frac{V(t)}{V_0}$$

Where α is an amplification factor, which for the current apparatus has been determined to be 0.0175. $V(t)$ is the transient signal size, is collected by the oscilloscope in millivolts and is proportional to the transient change in transmitted light intensity. A digital voltage meter monitors the dc voltage output of the preamplifier, V_0 , which is proportional to the total transmitted intensity before the flash.

CHAPTER 3

PROTEIN ADSORPTION ON NANOPOROUS TiO₂ FILMS AND THE PARAMETERS THAT CONTROL IT

3.1 Introduction

As I mentioned in chapter 1, many methods have been described for immobilising proteins on solid surfaces including, physical adsorption, covalent attachment, crosslinking, entrapment and/or encapsulation on matrixes or membranes (Scouten et al., 1995, Willner et al., 2000). Some of the parameters that have been described to influence protein-surface adsorption/interactions include: chemistry and morphology of the solid surface, pH, ionic strength, surface charge, isoelectric point of the surface and the protein, electrostatic interactions, temperature and hydrophobicity (Sadana et al., 1992).

The strong adsorption of redox proteins on electrode surfaces can result in attractive diffusionless systems for investigating biological electron transfer and for developing bioanalytical devices. It is important to be able to characterise the surface coverage, the orientation, the conformational and redox characteristics of the adsorbed proteins on electrodes. Optimising the conditions in order to get high protein loading with the immobilised proteins structurally and functionally intact is an advantage in the development of biosensors. Therefore, a detailed understanding of the protein adsorption process is required because in many cases the structure and thereby the function of protein changes upon adsorption.

In the present work attention is directed towards the ability of two heme proteins Cyt-c and Hb to undergo strong immobilisation on optically transparent, nanoporous TiO₂ films. Therefore, studies were conducted as a function of different immobilisation conditions by varying the pH and the ionic strength of the protein solutions used to immobilise the TiO₂ films. Also the ability of proteins with different surface charge, such as acetylated Cyt-c, to bind on the TiO₂ films was tested. Finally, stability trials

were conducted employing absorption spectroscopy to monitor any protein unbinding or denaturation.

The main aims are to obtain a more fundamental understanding of the binding of these well characterised electron carrier redox proteins on the TiO_2 films and to study the parameters, which might influence the binding.

3.1.1 Binding of dyes on TiO_2 films

It is known that TiO_2 has a high affinity for free carboxylate and phosphate groups (Murakoshi et al., 1995). Such molecular binding has been described to occur in order to fabricate devices, such as the solar cells, in which dyes are coated onto nanocrystalline TiO_2 surfaces (O'Regan et al., 1991). Binding is thought to involve a combination of electrostatic, covalent and hydrogen bonding forces. Evidence for covalent binding of carboxylate groups comes from resonance Raman spectra showing shifts in carbonyl bands for dyes attached to the Ti^{4+} of the TiO_2 film compared to that of the same dyes in solution (Fillinger et al, 1999). These results suggested that there are three possible coordination modes of the carboxylates of the dye on the TiO_2 film, which are unidentate (ester type C-O-Ti bond), bidentate chelating or bridging (i.e. two Ti^{4+} bound to one carboxylate).

3.1.2 Immobilisation proteins

Both cytochrome *c* and hemoglobin (see Table 3.1) were chosen for this research work because their structure, optical spectra, physical and electronic properties are well documented (Wood et al., 1984, Guo et al., 1991, Antonini et al., 1971), thus providing a basis for judging the suitability of nanoporous TiO₂ films for immobilising such metalloproteins.

Proteins	Molecular Weight	Dimensions (nm ³)	IEP	Function
Cytochrome <i>c</i>	12327	2.5 x 3.2 x 3.7	~9.8	redox
Hemoglobin	64550	6.4 x 5.5 x 5.0	~7.2	O ₂ transporter

Table 3.1: Proteins used for immobilisation on TiO₂ films and their molecular parameters

3.1.3 Cytochrome *c*

Cytochrome *c* (Cyt-*c*) is a peripheral membrane protein that is loosely bound to the outer surface of the inner mitochondrial membrane. It is a relatively small (12 kDa) water soluble heme protein, has a net charge of +7.5 in its oxidised state (FeIII) and a midpoint redox potential of +260 mV vs NHE (Hettinger et al., 1965). Each protein molecule is roughly spherical and has a diameter of ~32.4 Å (Guo et al., 1995). Its isoelectric point is 10 and it is very stable in a wide pH range (Collinson et al., 1992).

Cyt-*c* consists of a single polypeptide chain of 104 amino acid residues folded in a globular, mostly helical structure, which creates a heme binding pocket. One heme edge is relatively exposed to the environment (see figure 3.1). Cyt-*c* is a redox-active protein which contains a single heme group that reversibly alternates between its Fe(II) and Fe(III) oxidation states during electron transport.

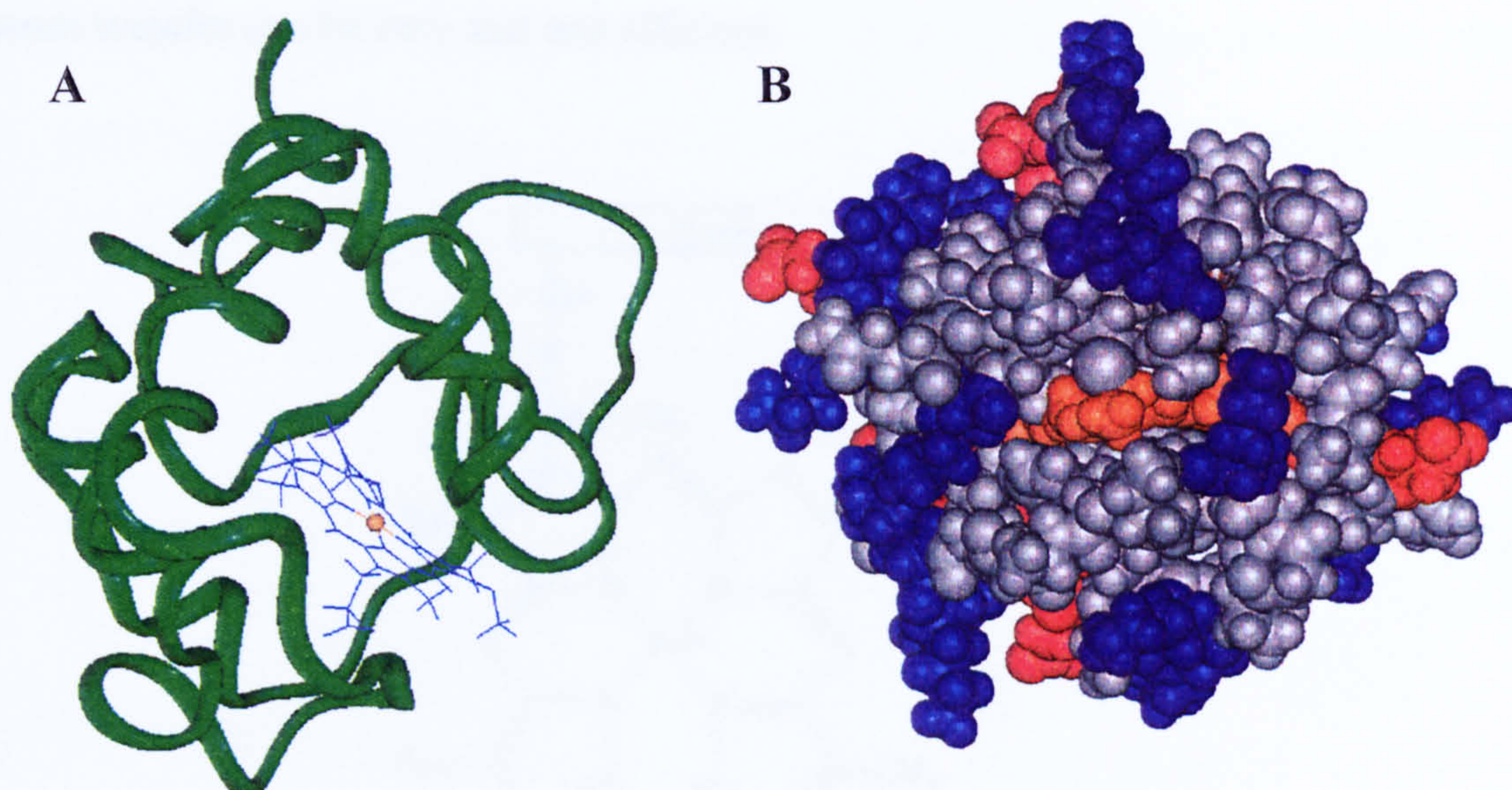


Figure 3.1: (A) Ribbon diagram of cytochrome c showing the position of the heme (blue) and the iron (orange). (B) The molecular surface and charge distribution in Cyt-*c*. The surface is colored according to charge. Negatively charged groups (Asp and Glu) are red and positively charged groups (Lys and Arg) are blue. Uncharged groups are grey and heme is orange.

The heme consists of a porphyrin ring coordinated with the redox active iron atom. The Cyt-*c* heme is not a cofactor, it is covalently attached to the protein's polypeptide chain by thioether bridges through two cysteine residues (see figure 3.2) (Dickerson et al., 1975). The iron is bound by two axial ligands, a histidine and a methionine in both Fe(II) and Fe(III) oxidation states. The actual structure of both states are similar, with only a small reorganisational barrier to electron transfer. Therefore, electron transfer can be very fast and efficient.

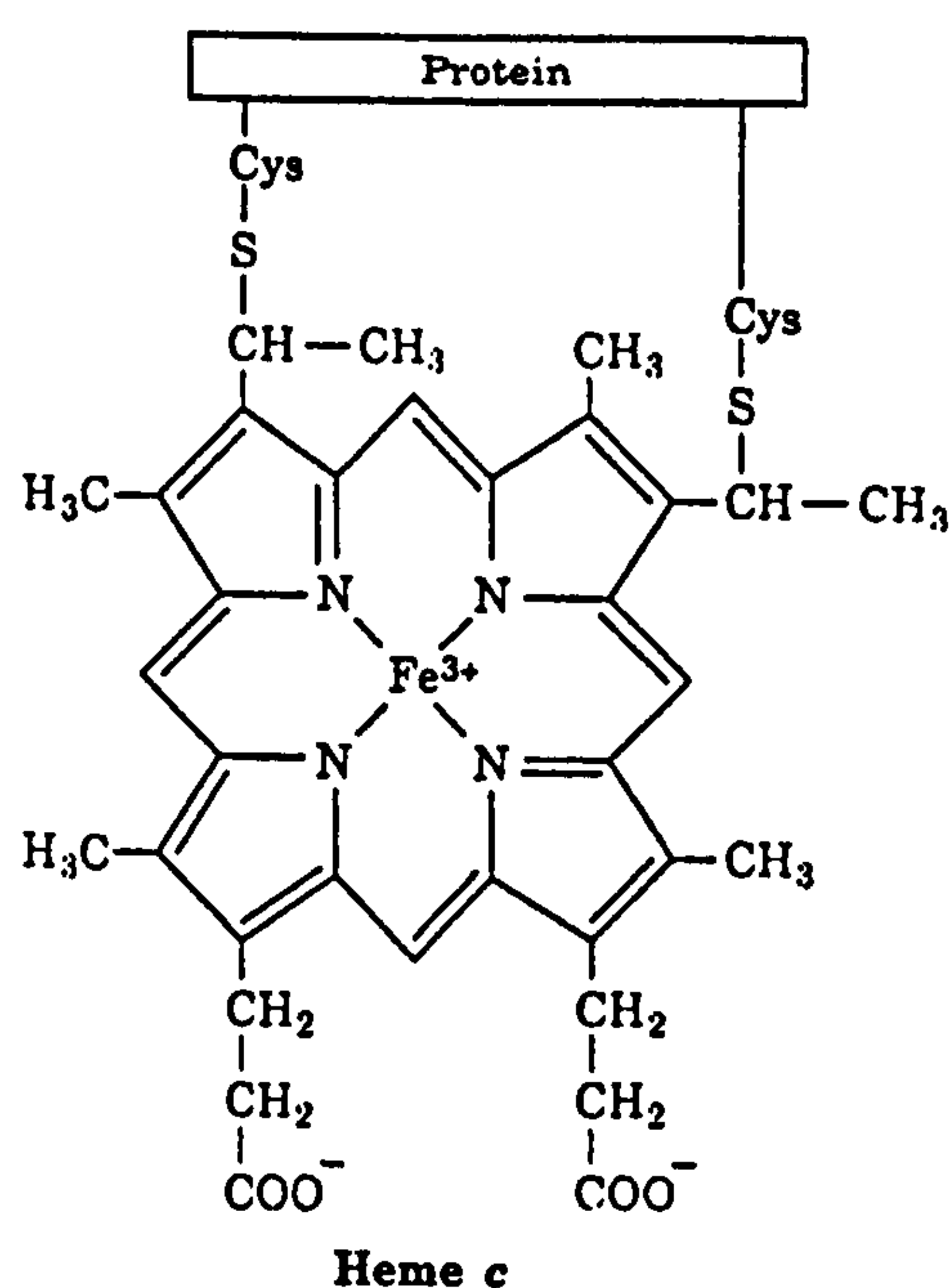


Figure 3.2: The chemical structure of the heme group contained in cytochrome *c*.

Both the reduced and the oxidised forms of Cyt-*c* have characteristic absorption spectra, which reflect the conformation or structure of the protein. The heme group of reduced Cyt-*c* has an intense absorption band in the UV, the Soret band at 415 nm and two absorption bands in the visible spectrum, α at 521 nm and β at 550 nm (Wood et al., 1984). Oxidation of the heme causes a shift of the Soret band to 410 nm and the α

and β bands disappear giving rise to broader band at 530 nm. Changes in the protein environment induce small structural changes, which appear as shifts in the absorption spectrum and might cause denaturation of the protein.

Figure 3.1B shows the charge distribution on the surface of Cyt-*c*. Several invariant lysine and arginine residues lie in a ring around the exposed edge of the otherwise buried heme group. Therefore the adsorption of Cyt-*c* on the negatively charged nanoporous TiO₂ films should occur with the heme facing the surface of the film, allowing direct electron transfer to occur. The likely minimum distance for electron transfer between the Cyt-*c* and the TiO film is estimated to be ~3.3-3.5 Å.

3.1.4 Hemoglobin

Hemoglobin is an [$\alpha(2):\beta(2)$] tetrameric, large (64 kDa) heme protein found in erythrocytes where it is responsible for binding O₂ in the lung and transporting it throughout the body for use in aerobic metabolic pathways (Antonini et al., 1971). The Hb molecule (monomer) is spherical, has a diameter of 55 Å and consists of two α chains (141 residues each) and two β chains (146 residues each) packed together in a tetrahedral arrangement.

One heme is bound noncovalently to each polypeptide chain of Hb. The heme is responsible for the characteristic red color of blood and is the site at which each globin monomer binds one molecule of oxygen. The heme groups are located in crevices near the exterior of the molecule, one in each subunit (see Figure 3.3). Each of the four

hemes resides in a crevice between two helices (E, F) with the its polar propionate groups at the protein surface. The surface of Hb is polar and the interior non-polar and its isoelectric point is 7 (Antonini et al., 1971). The midpoint redox potential of Hb is +150 mV vs NHE (Benerjee et al., 1969).

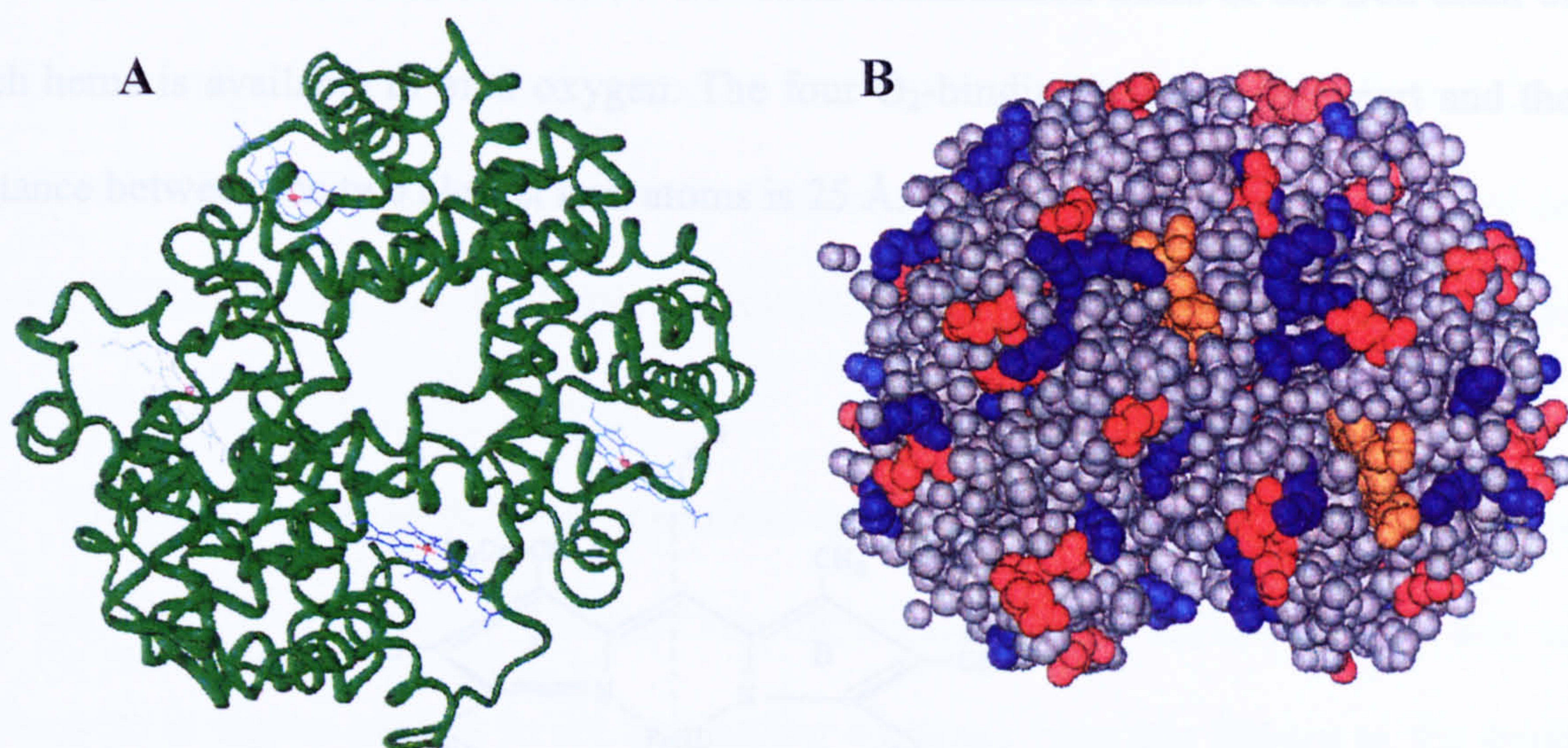


Figure 3.3: (A) Ribbon diagram of Hb showing the position of the four hemes (blue) and the four irons (orange). (B) The molecular surface and charge distribution in Hb. Surface is colored according to charge. Negatively charged groups (Asp and Glu) are red and positively charged groups (Lys and Arg) are blue. Uncharged groups are grey and the hemes are orange.

The heterocyclic ring system of heme is a porphyrin derivative; it consists of four pyrrole rings (letters A-D in figure 3.4) linked by methene bridges. The porphyrin in heme, with its particular arrangement of four methyl, two propionate, and two vinyl substituents, is known as protoporphyrin IX. Heme then is protoporphyrin IX with a centrally bound iron atom (see Figure 3.4). The iron atom in the ferrous deoxygenated Hb is 5-coordinated by a square pyramid of N atoms: four from the porphyrin and one from a histidine side chain of the Hb. The sixth coordination bond of the iron atom of each heme is available to bind oxygen. The four O₂-binding sites are far apart and the distance between the two closest iron atoms is 25 Å.

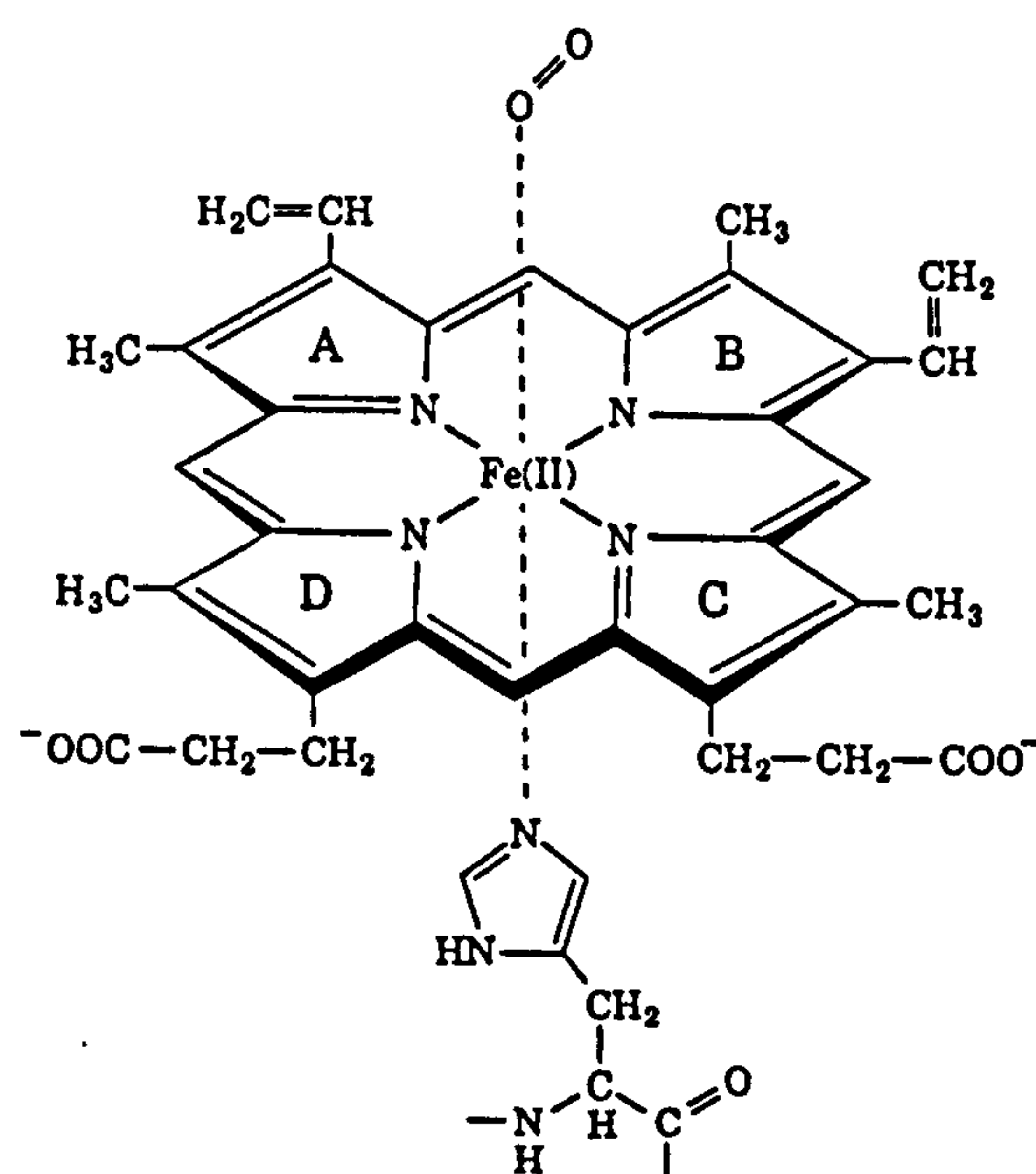


Figure 3.4: Fe(II)-heme (ferroprotoporphyrin IX) shown liganded to His and O₂ as it is in oxygenated Hb. The heme is a conjugated system so that although two of its Fe-N bonds are coordinate covalent bonds, all of the Fe-N bonds are equivalent. The pyrrole ring lettering scheme is also shown.

Upon oxygenation, the oxygen binds to the Fe(II) on the opposite side of the porphyrin ring from the histidine ligand so that the Fe(II) is octahedrally coordinated. Certain small molecules, such as CO and NO coordinate also to the sixth liganding position of the Fe(II) in Hb with much greater affinity than does O₂. This accounts for the highly toxic properties of these substances.

Finally, the Fe(II) of Hb can be oxidised to Fe(III) to form methemoglobin (metHb). MetHb does not bind O₂, a fact that interferes with the physiological role of the protein. The oxidation reaction results in a transition from the non-ligated five coordinated Hb to the ligated six-coordinated protein. A water molecule instead of an oxygen molecule occupies the sixth coordination site. The brown color of the dried blood is that of metHb.

The visual spectrum of Hb is essentially determined by the iron-porphyrin moiety and the spectral differences between the common protein derivatives are due to differences in ligand binding to the iron and/or a change from the ferrous to the ferric state. The absorption band maxima of the ferric and ferrous derivatives of Hb are shown on table 3.2 (Antonini et al., 1971).

Protein (solution)	Absorption band maxima (nm)
Met (Fe ^{III}) Hb	406, 498, 626
Deoxy (Fe ^{II}) Hb	428, 558
Oxy (Fe ^{II}) Hb	415, 541, 576
CO (Fe ^{II}) Hb	419, 540, 569
NO (Fe ^{II}) Hb	418, 538, 568

Table 3.2: Absorption band maxima of Hb, both unbound and ligated to O₂, CO and NO.

Figure 3.3B shows the charge distribution on the surface of Hb. The positive patches next to the exposed edges of the hemes should position Hb on the negatively charged TiO₂ film in an orientation which allow one or two of the hemes to come in direct contact with the surface of the film, making direct electron transfer possible. Therefore, the likely minimum distance for electron transfer between Hb and the TiO₂ film is estimated to be ~3.7-3.9 Å.

3.2 Experimental Conditions

3.2.1 Protein immobilisation

Nanoporous TiO₂ films were prepared from an aqueous suspension of ~ 15 nm anatase TiO₂ colloids as described in chapter 2. Protein solutions were also prepared as described in chapter 2. Immobilisation was achieved by the immersion of 1-cm² TiO₂ films in protein solutions at 4° C for 1 - 30 days depending upon protein and immobilisation conditions. The aqueous immobilisation solutions comprised the protein of interest *Cyt-c* (4-25 µM), *Hb* (3-30 µM), acetylated *Cyt-c* (15 µM) and 10 mM NaH₂PO₄ buffer, with the pH adjusted to a value of 7 using NaOH. All solutions were prepared in distilled de-ionized water. Protein adsorption onto the TiO₂ films was monitored by recording the UV/visible absorption spectra of the protein immobilised films at room temperature on a Shimadzu UV-1601 spectrophotometer. Contributions to the spectra from scatter and absorption by the TiO₂ film alone were subtracted by the

use of protein free reference films. Prior to all spectroscopic measurements, films were removed from the immobilisation solution and rinsed in buffer solution to remove non-immobilised protein.

The same procedure was also used for the immobilisation of Cyt-c on 1cm² nanocrystalline ZnO films of 4 µm thickness.

3.2.2 Effect of pH

In order to examine the effect of pH on the adsorption of Cyt-c and Hb on the TiO₂ films, Cyt-c and Hb solutions of 20 and 18 µM concentration respectively were prepared in phosphate buffer at pH values varying between 5.5 and 10 and 1-cm² TiO₂ films were immersed therein at 4° C. Absorption spectra was monitored over a period of 30 days.

3.2.3 Effect of ionic strength

Cyt-c solutions (20 µM) of different NaCl concentrations (5-700 mM) were prepared in phosphate buffer at pH 7 in order to check the effect of the salt during the adsorption process. The absorption spectra of the Cyt-c/TiO₂ films were monitored over a period of 30 days.

3.2.4 Desorption studies

Following adsorption of Cyt-c and Hb on the nanoporous TiO_2 films, the immobilised films were immersed in protein free buffer solution of pH 7 and any protein desorption was monitored over time. In addition the effect of NaCl on the stability of the Cyt-c/ TiO_2 films was examined by incorporating 0.01, 0.1 and 0.5 M of NaCl respectively in the 4 ml 10 mM phosphate buffer solution of pH 7 where the Cyt-c/ TiO_2 films were immersed after the adsorption process was complete. Any Cyt-c desorption was monitored over a period of 30 days.

3.3 Initial Studies of Binding

3.3.1 Results

3.3.1.1 Protein adsorption on TiO₂ films

Cyt-*c* and Hb may be adsorbed successfully to nanoporous TiO₂ films by immersion of such films in aqueous protein solutions at 4 °C (Topoglidis et al., 2000). The optical transparency of the TiO₂ allows the adsorption process to be monitored by UV-Vis absorption spectroscopy.

Figure 3.5 shows absorption spectra obtained for a Cyt-*c* / TiO₂ and a Hb / TiO₂ nanoporous film. Non-immobilised proteins were removed prior to all spectroscopic measurements by rinsing the films in buffer solution. Also shown is the absorption spectrum of a TiO₂ film prior to protein immobilisation.

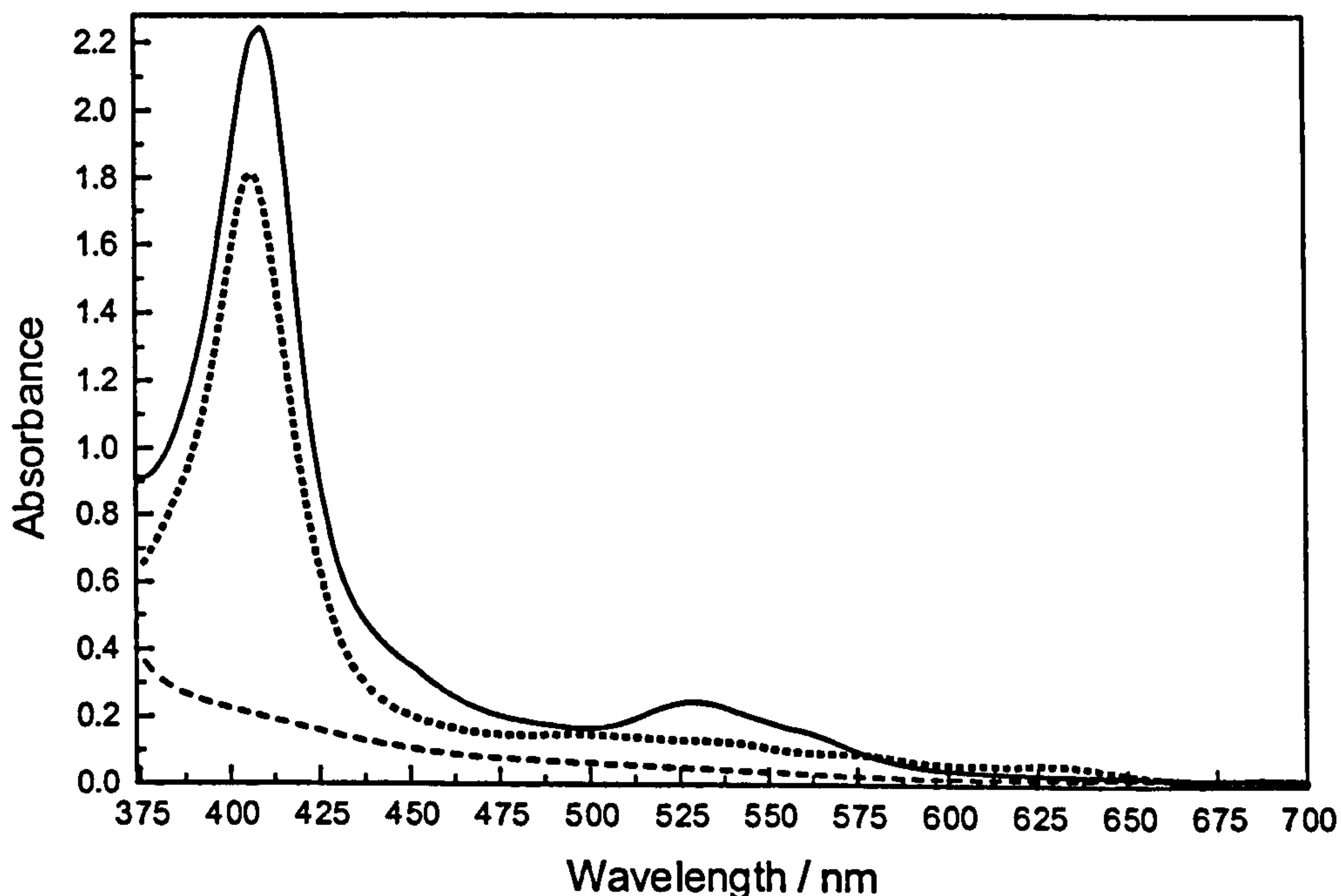


Figure 3.5: Absorption spectra of a nanoporous TiO₂ film before (----) and after the immobilisation of Cyt-*c* (—) and Hb (.....). Spectra uncorrected for sample scatter.

Bare TiO_2 films are transparent and colourless in the visible region, showing a characteristic absorption increase below 400 nm due to the onset of TiO_2 band gap excitation. Adsorption of Cyt-c or Hb on TiO_2 films, results in orange and brown coloration of the films respectively. The absorption spectra of Cyt-c and Hb on 8 μm thick nanoporous TiO_2 films shows the characteristic absorption heme bands at 410 and 529 nm for Fe(III)Cyt-c and at 406 nm of oxidised met Fe(III)Hb in good agreement with the solution spectra of these proteins and indicating that the immobilisation of the proteins in the films does not cause denaturation (Stellwagen et al., 1968 and Antonini et al., 1971). A blue shift in the Soret band of Cyt-c or metHb would be a key indicator of protein denaturation after adsorption. Denaturation of Cyt-c and metHb causes a blue shift of the Cyt-c Soret peak to 395 nm and of the metHb Soret peak to 402 nm, spectroscopic characteristics clearly not observed during the protein adsorption process. In addition, the weak 695 nm absorption band of Cyt-c was retained after adsorption, indicating that the bond between the heme iron and the sulphur atom of Met 80 remained intact (Dickerson et al., 1975).

Figures 3.6 and 3.7 show the adsorption of Fe(III) Cyt-c and metHb on 4 and 8 μm thick nanoporous TiO_2 films at pH 8 respectively. The insert of each figure shows the time course for the adsorption, monitoring the appearance of the heme Soret absorption at 410 and 406 nm for immobilised Fe(III)Cyt-c and met Fe(III)Hb respectively.

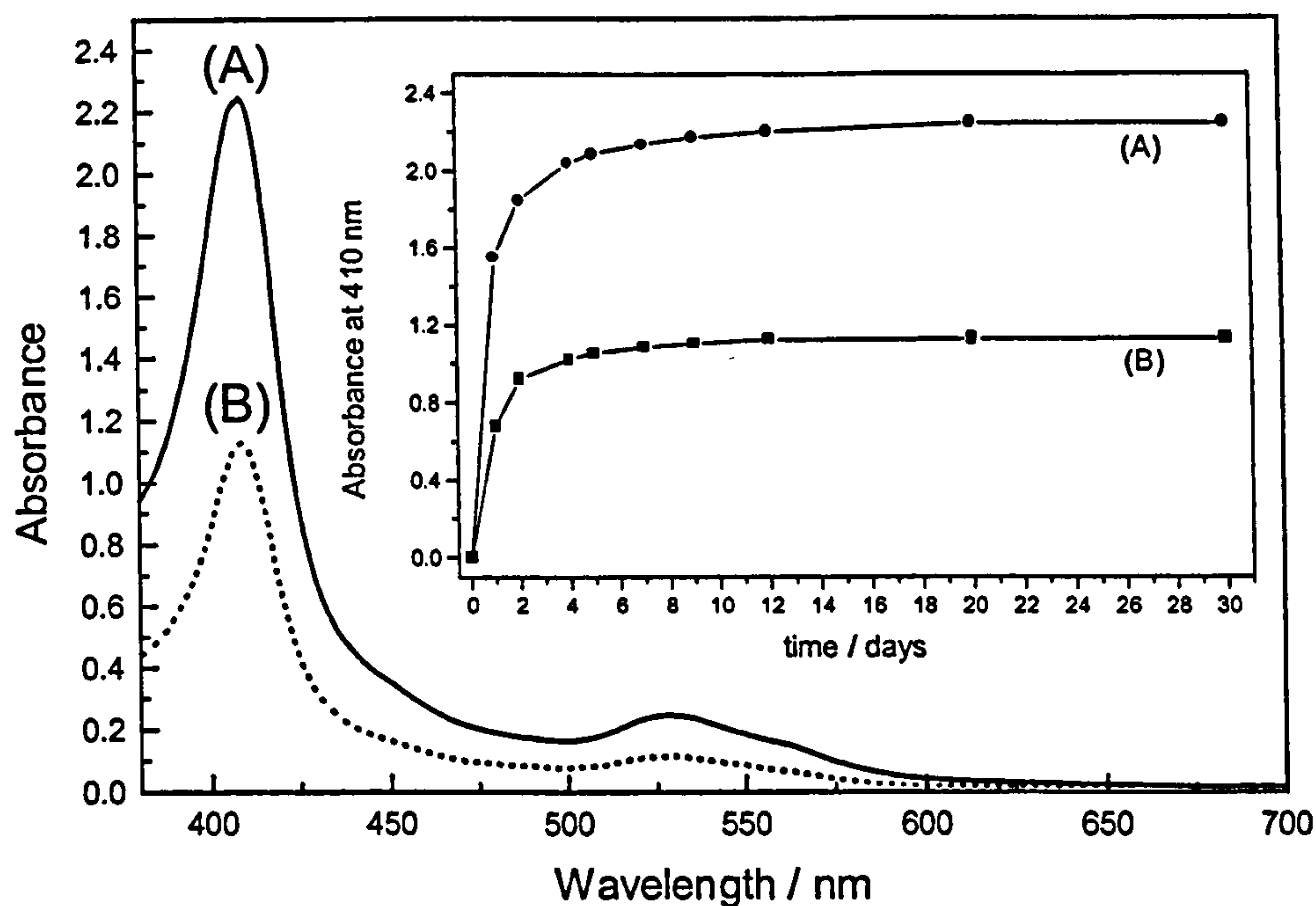


Figure 3.6: Absorption spectrum of a (A): 8 and (B): 4 μm thick nanoporous TiO₂ film after the immobilisation of *Cyt-c*. The inset shows the increase in absorbance at 410 nm of each *Cyt-c*/TiO₂ film over a period of 30 days until saturation was achieved.

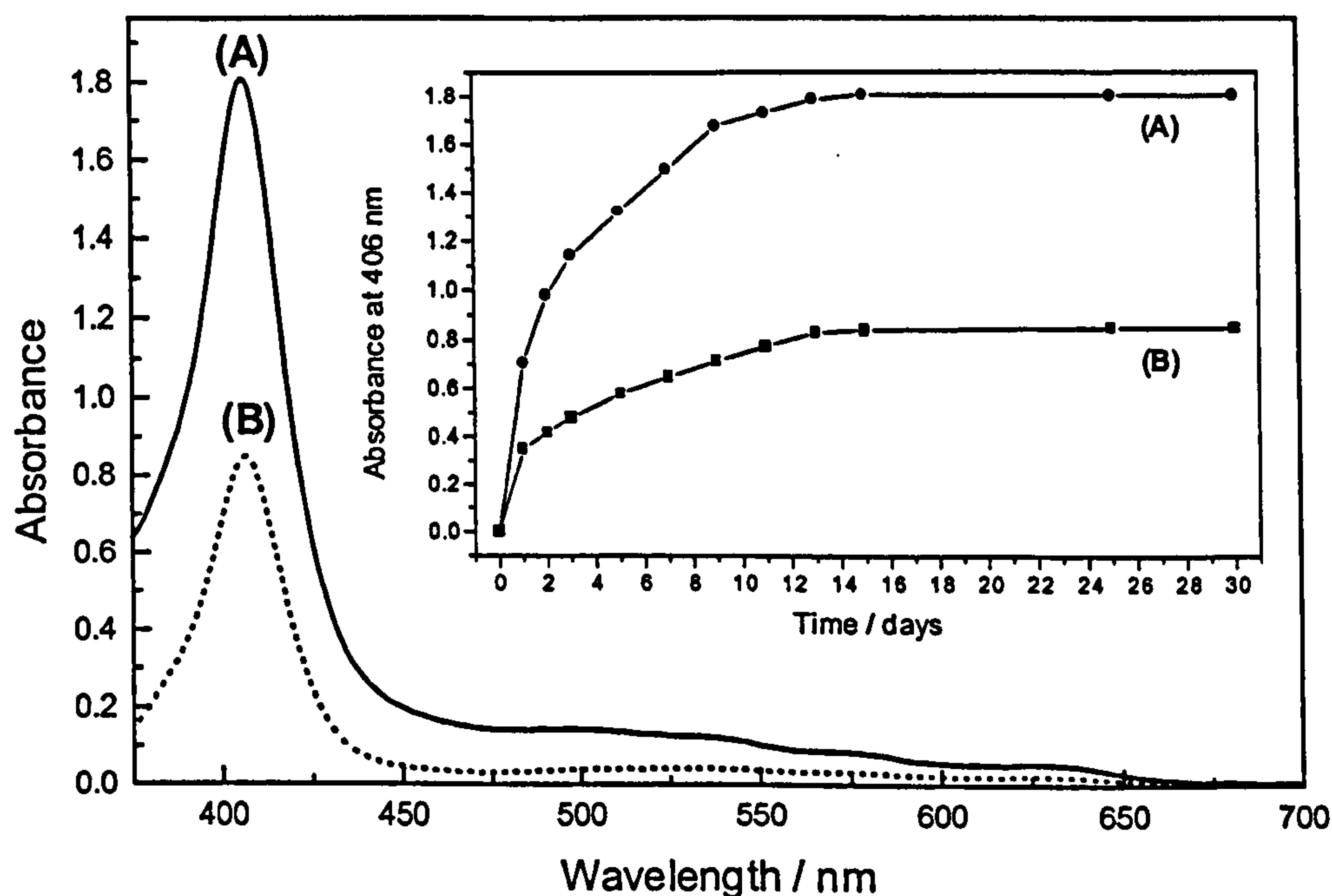


Figure 3.7: Absorption spectrum of a (A): 8 and (B): 4 μm thick nanoporous TiO₂ film after the immobilisation of MetHb. The inset shows the increase in absorbance at 406 nm of each MetHb/TiO₂ film over a period of 30 days until saturation was achieved.

From the observed absorption spectra of the immobilised Cyt-c, corrected for the absorption by the TiO_2 film and the conducting glass, and employing an extinction coefficient of $106,000 \text{ M}^{-1} \text{ cm}^{-1}$ at 410 nm for Fe(III) Cyt-c (Wood et al., 1984), a protein loading at saturation of 19 nmoles for the 8 μm thick, 1 cm^2 film was obtained. This high protein loading, corresponding to a loading 850 times greater than monolayer coverage of a flat electrode surface (assuming a cross-sectional area for Cyt-c of 7 nm^2), results from adsorption within the film pores.

Similarly, from the observed absorption spectra of the immobilised Hb and employing an extinction coefficient of $164,000 \text{ M}^{-1} \text{ cm}^{-1}$ at 406 nm for metHb (Antonini et al., 1971), a protein loading at saturation of 11 nmoles for the 8 μm thick, 1 cm^2 film was obtained. This is also a high protein loading, corresponding to a loading 575 times greater than monolayer coverage of a flat electrode surface (assuming a cross-sectional area for Hb of 24 nm^2) and resulting from adsorption within the film pores.

Comparison with dye-binding studies suggests that Cyt-c and Hb achieve a $70 \pm 10 \%$ and a $60 \pm 10\%$ coverage of the internal surface area of the film available for dye binding. The somewhat lower binding achieved with the proteins is most probably due to the larger dimensions of Cyt-c (diameter $\sim 3 \text{ nm}$) and Hb (diameter $\sim 5.5 \text{ nm}$) which will prevent access to smaller pores of the film.

Finally, further reference to figures 3.6 and 3.7 shows that both Cyt-c and Hb adsorption are proportional to film thickness, with the use of 4 μm film resulting in a 2 fold reduction in the amount of each protein adsorbed. This is consistent with uniform protein adsorption throughout the pores of the film, suggesting that the TiO_2 film structure is such as to allow penetration of protein through its entire depth.

3.3.1.2 Cyt-c adsorption on nanocrystalline ZnO films

Cyt-c was also immobilised on another metal oxide, ZnO, successfully. Due to the limited number of nanocrystalline ZnO films that were available during the project, only a very basic adsorption study was carried out.

Blank ZnO films are, like TiO₂ films, transparent and colourless, displaying the fundamental absorption edge of anatase (band gap 3.2 eV) in the ultraviolet region (Hagfeldt et al., 1995). Figure 3.8 shows the absorption spectra of a blank ZnO and a blank TiO₂ respectively. The ZnO film shows a characteristic absorption increase below 400 nm due to the onset of ZnO band gap excitation.

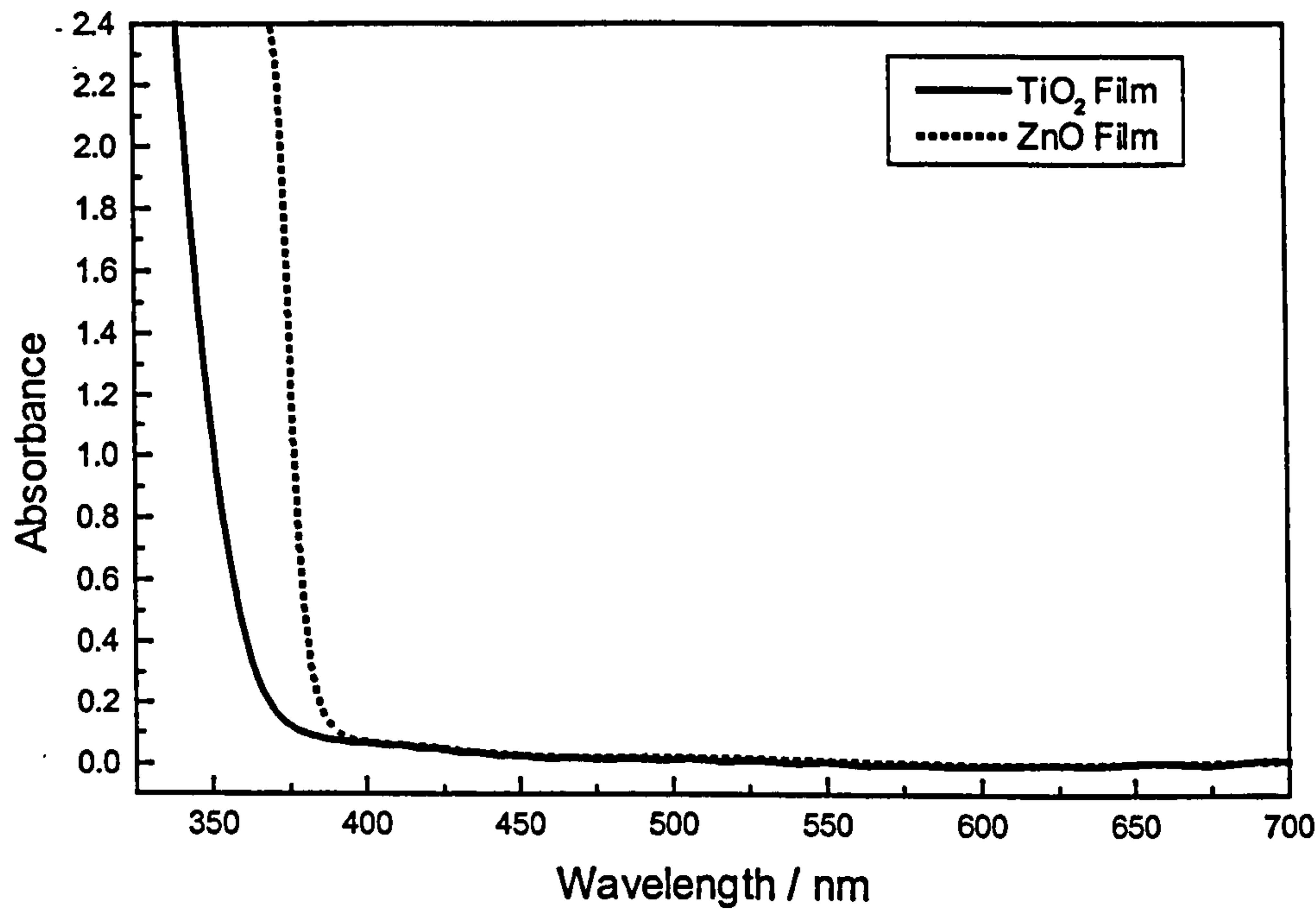


Figure 3.8: Absorption spectra of a blank TiO₂ film and a blank ZnO film.

Cyt-c was adsorbed successfully to 4 μm nanocrystalline ZnO films by immersion of such films in 20 μM aqueous protein solution at 4 $^{\circ}\text{C}$. The optical transparency of the ZnO films allows the adsorption process to be monitored by UV-Vis absorption spectroscopy. Figure 3.9 shows the absorption spectra for a Cyt-c/ZnO film and the inset of the figure shows the time course for the adsorption monitoring the appearance of the heme Soret absorption at 410 nm for Fe (III) Cyt-c. The spectrum of the Cyt-c/ZnO film is in good agreement with the solution spectrum of Cyt-c indicating that the adsorption of Cyt-c in the ZnO film does not cause denaturation. Cyt-c adsorption saturates after approximately 15-16 days, being 35% complete within the first day.

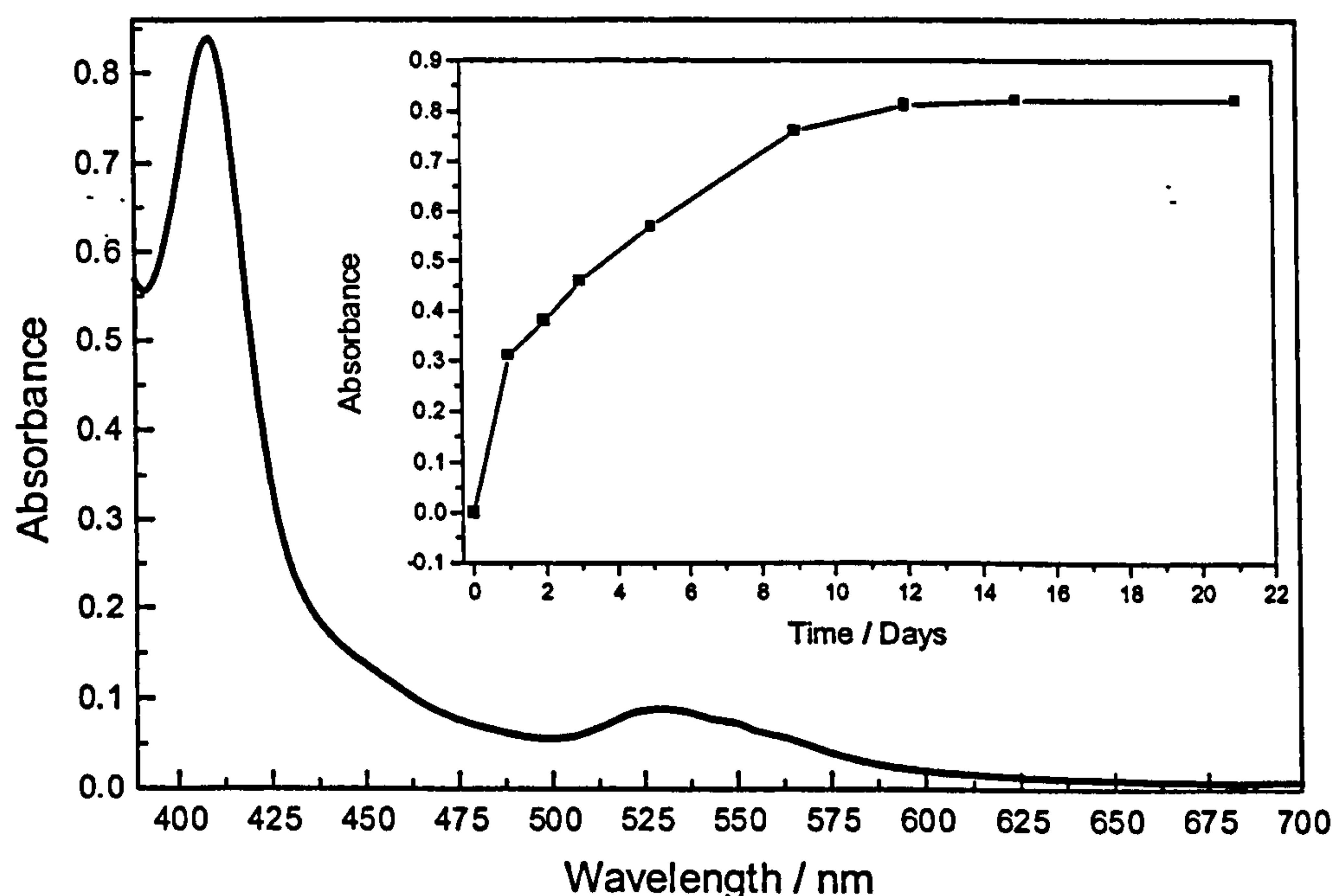


Figure 3.9: Absorption spectrum of a 4 μm thick nanocrystalline ZnO film after the immobilisation of Cyt-c. The inset shows the increase in absorbance at 410 nm of the Cyt-c/TiO₂ film over a period of 22 days until saturation was achieved.

By using the same method used earlier to estimate the Cyt-c and Hb loadings on the TiO₂ films, it was possible to obtain a Cyt-c loading at saturation of 8 nmoles for the 4 µm thick, 1 cm² ZnO film. This loading is 30% less than the loading (11 nmoles) obtained for the 1 cm² TiO₂ film of the same thickness.

3.3.1.3 Improved Stability

It is interesting to note that the Cyt-c solution used to sensitise the TiO₂ films denatured after a period of one month at 4 °C. A reference solution of Cyt-c was used to determine the influence of buffer solution and storage time on protein stability. Figure 3.10 confirms that the Soret band of the one month old Cyt-c solution changes from $\lambda_{\text{max}} = 410 \text{ nm}$ to $\lambda_{\text{max}} = 396 \text{ nm}$ (a spectral blue shift of 14 nm was monitored relative to that of native protein in buffer solution) and these spectral changes represent denaturation of Cyt-c. On the other hand the immobilised Cyt-c retained its native conformation even 3 months after immobilisation. The film remained transparent and the characteristic red colour of Cyt-c was clearly visible. Similar results (not shown here) have been obtained for Hb as well. An analogous improvement in protein stability upon immobilisation has also been reported by Liu et al., 1999. According to their studies, proteins showed very good conformational stability after long-term sol-gel encapsulation. The improved stability was attributed to the fact that when the pore size is sufficiently small to fully constrain the protein molecule, no further large-scale conformational changes can occur.

The very good conformational stability of the immobilised Cyt-c and Hb in my studies suggests that the nanopores of the TiO_2 films at least partially constrain the structure of the protein whilst apparently not interfering with its function. Although buffer solution is usually a suitable environment for carrying proteins without affecting their native conformations, the mobility and flexibility of proteins in such solution is ultimately detrimental to their long-term storage. An immobilised protein appears to be more stable and protected from aggregation and unfolding in a structurally solid environment which is provided by a pore space that is close to protein molecules dimensions.

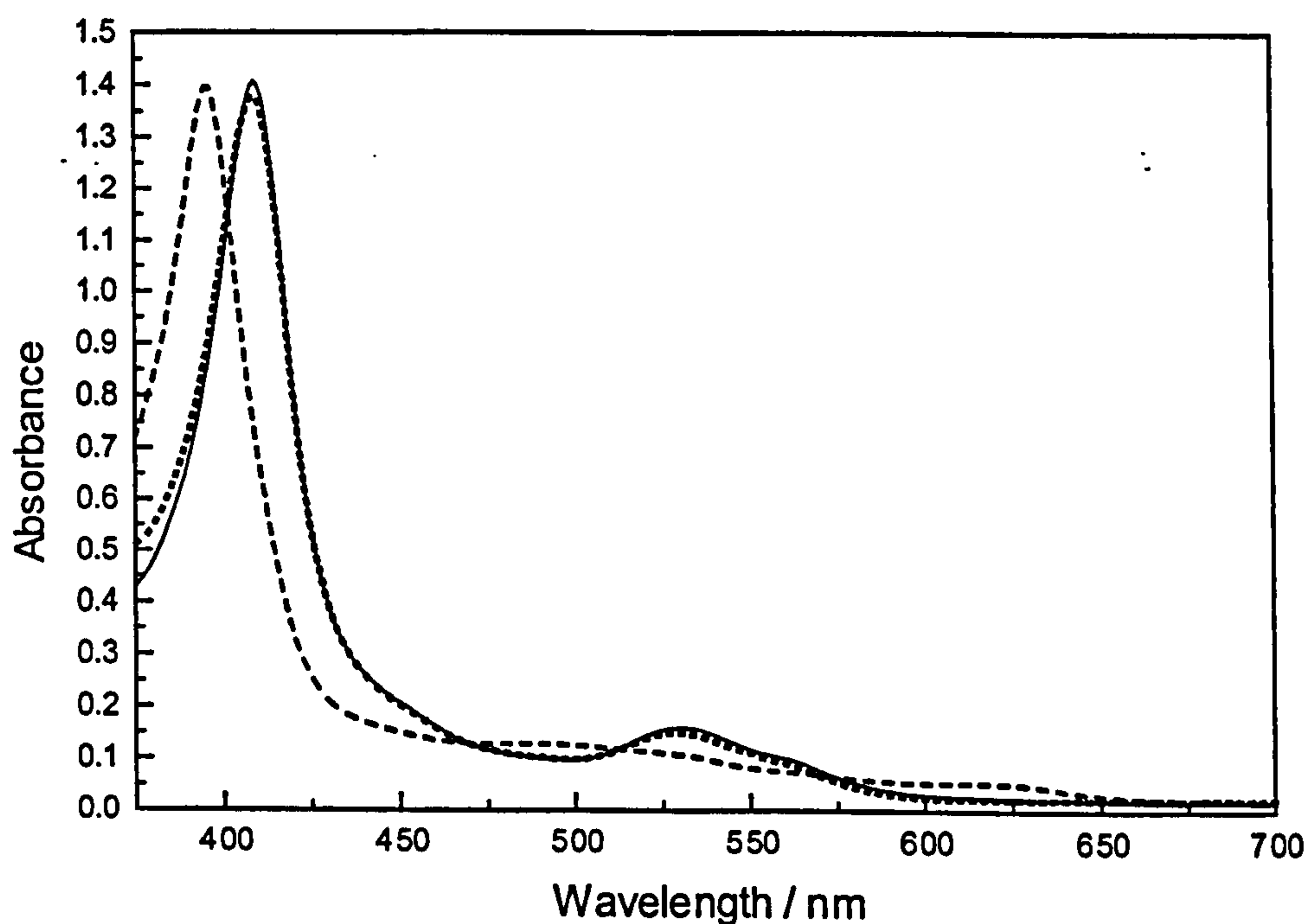


Figure 3.10: Absorption spectra of fresh Cyt-c solution (—), of a 1 month old Cyt-c/ TiO_2 film (.....) and of the Cyt-c solution used for immobilisation after 1 month (----).

3.3.2 Discussion

3.3.2.1 Protein adsorption

Prior to my own studies, as reported here, there had been no study of immobilisation of proteins upon a nanoporous metal oxide films. I have shown here that Cyt-*c* and Hb were immobilised successfully on nanoporous TiO₂ films by direct physical adsorption at 4 °C without the modification of the protein structure or the surface of the TiO₂ film (Topoglidis et al., 1998 and 2000). In addition Cyt-*c* was immobilised successfully on nanocrystalline ZnO films. Protein adsorption occurs at surface coverages up to 850 times greater than a monolayer and with good retention of protein structure. The TiO₂ films described here are 20 times thicker than multilayer films of protein and polyions described previously (Lvov et al., 2000) and the amount of protein adsorbed is 100 times more. This is of paramount importance if the biological properties of the immobilised proteins, such as their redox activity and sensitivity to ligands, are to be exploited. It is also apparent that protein adsorption occurs uniformly through the TiO₂ film, the amount of protein for an 8 µm film being twice that for a 4 µm film. Finally, the film fabrication and protein immobilisation steps were separated and therefore immobilisation was conducted under benign conditions of temperature, pH and solvent, therefore avoiding denaturation or inactivation of the adsorbed proteins. In addition, the large surface area of the TiO₂ film with pores of similar dimensions to a protein allows high levels of protein adsorption without loss of protein structure since the protein is stabilised and constrained by the pores of the film (Topoglidis et al., 2001).

3.3.2.2 Modelling the protein diffusion kinetics

Cyt-c shows significantly faster immobilisation in the TiO₂ films than Hb as illustrated in the insets of figures 3.6 and 3.7. Cyt-c adsorption saturates after approximately 12 days, being 60 % complete within the first day. Met Hb adsorption saturates after approximately 14 days, being 40 % complete within the first day. In both cases the rate of protein adsorption is probably limited by protein diffusion times through the pores of the film.

In order to understand the kinetics of protein diffusion into the nanoporous TiO₂ film, a theoretical model was applied. The kinetics of diffusion of protein molecules (Cyt-c or Hb) into the nanoporous structure of the TiO₂ film can be expressed by Fick's first law of diffusion (equation 1) which describes the net flow of species from a region of high concentration (bulk solution) to a region of low concentration (film pores) (McGregor et al., 1974):

$$F = -DA\left(\frac{dc}{dx}\right) \quad (1)$$

Where F is the diffusion flux, A is the surface available for diffusion, D is the diffusion coefficient and (dc/dx) is the concentration gradient. For such systems the rate of equilibration of protein concentration is expected to follow equation 2:

$$c_{\text{Immob}} = c_{\text{Immob}}^{\text{Sat}}(1 - e^{-t/\tau}) \quad (2)$$

Where c_{Immob} is the concentration of protein on the TiO₂ at time t, $c_{\text{Immob}}^{\text{Sat}}$ is the concentration of protein on the film at saturation and τ is the time constant for equilibration. Since the UV/Vis absorbance of the adsorbed protein on the film is

directly proportional to the concentration of adsorbed protein, absorbance can be substituted for concentration in equation 2 to give:

$$Abs_{immob} = Abs_{immob}^{Sat} (1 - e^{-t/\tau}) \quad (3)$$

The above form (equation 3) is now a simple inverse exponential kinetic equation that is commonly used in the analysis of mass transfer between phases (McGregor et al., 1974). The fit of equation 3 to adsorption of Cyt-c and Hb on TiO_2 films is shown in figures 3.11a and 3.11b respectively.

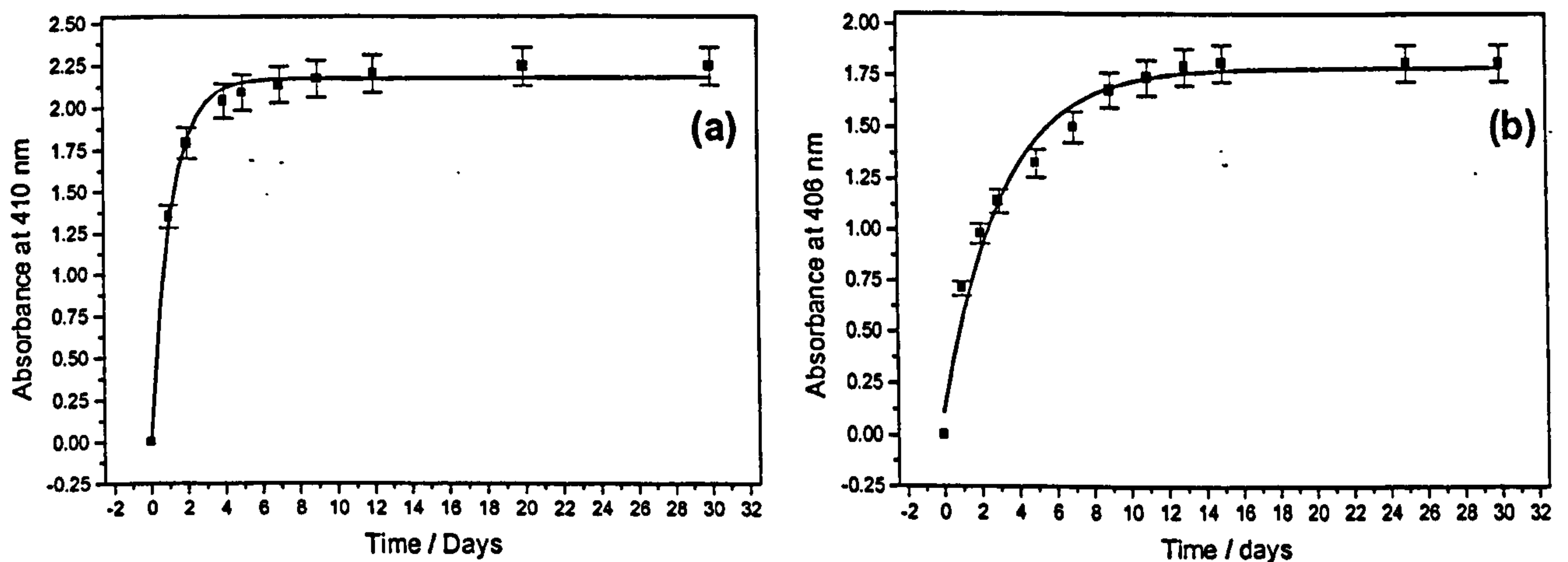


Figure 3.11: Adsorption kinetics for (a) Cyt-c and (b) Hb on 8 μm thick nanoporous TiO_2 films over a period of 30 days. Solid lines show the best fit to Fick's law.

A good fit is obtained for both proteins giving $t_{1/2}$ values of 1 and 3 days for the Cyt-c/ TiO_2 and Hb/ TiO_2 films respectively. The diffusion time, which is 3 times slower for Hb than for Cyt-c, correlates with the fact that Hb has a much larger hydrodynamic radius than Cyt-c (Bonicontró et al., 2000). In solution the diffusion coefficient, D , is

inversely proportional to the hydrodynamic radius, (the hydrodynamic radii of Hb and Cyt-*c* are 3.5 and 1.7 nm respectively) and one would therefore expect Cyt-*c* to diffuse twice as fast as Hb. In fact, Cyt-*c* diffuses three times faster in reasonable agreement with the theoretical calculation. The slightly larger ratio of 3 obtained experimentally may be the result of smaller pores hindering the rate of diffusion of the larger Hb molecule through the film (Topoglidis et al., 2001).

The same model (equation 3) can be used to explain the diffusion kinetics of Cyt-*c* to the ZnO film. The fit of equation 3 to adsorption of Cyt-*c* on a 4 μm TiO₂ and a 4 μm ZnO film is shown in figures 3.12a and 3.12b respectively.

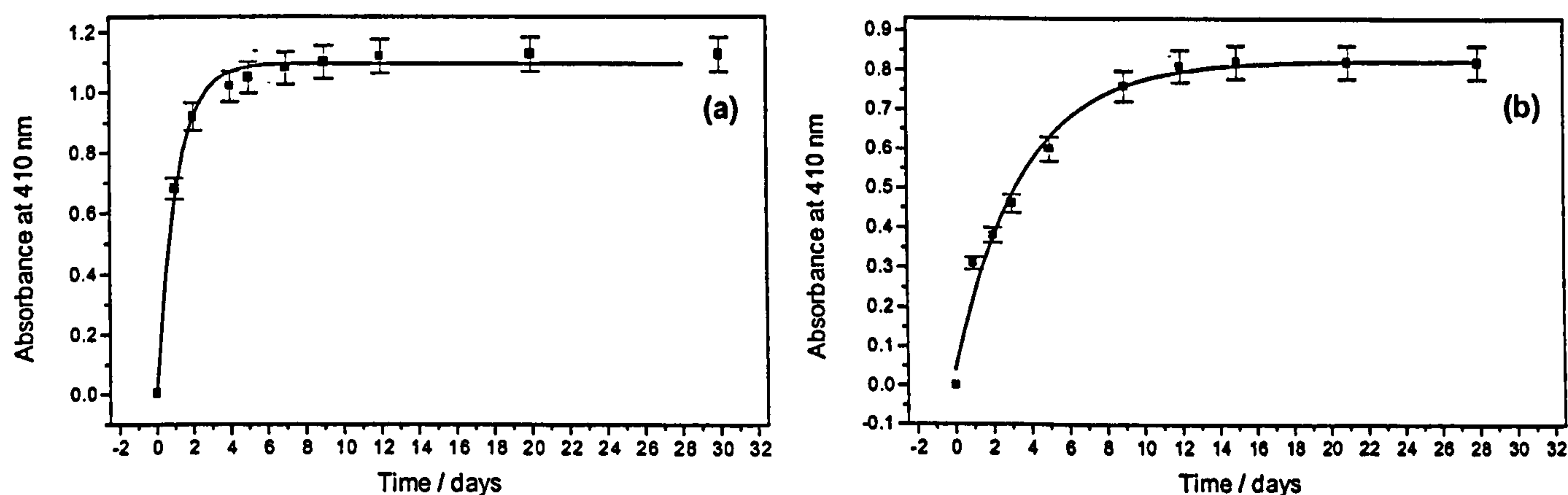


Figure 3.12: Adsorption kinetics for Cyt-*c* (a) on a 4 μm thick TiO₂ film and (b) on a 4 μm thick ZnO film over a period of 30 days. Solid lines show the best fit to Fick's law.

Again a reasonable good fit is obtained for the diffusion of Cyt-*c* on both films. The $t_{1/2}$ were 1 and 3.5 days for the Cyt-*c*/TiO₂ and Cyt-*c*/ZnO films respectively. The diffusion time of Cyt-*c* is 3.5 times slower into the ZnO film than into the TiO₂ film.

These results correlate well with the fact that according to the SEM of the ZnO film (see figure 3.13) and the SEM TiO₂ presented in chapter 1, the ZnO film is less porous and the size of its pores smaller than the ones of the TiO₂ film. Therefore the protein loading is slower.

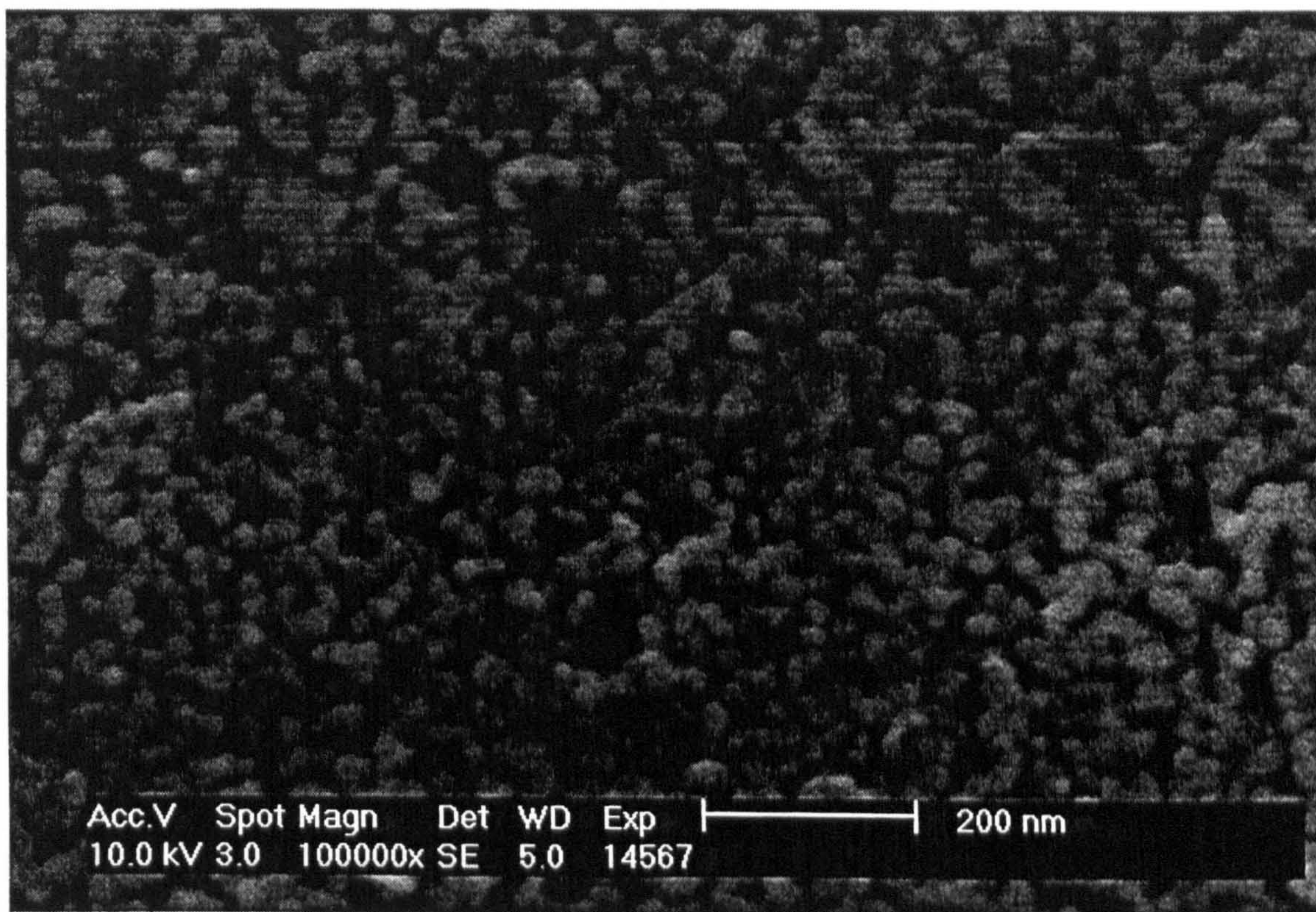


Figure 3.13: SEM picture of a 10 μm thick nanocrystalline ZnO film.

Another interesting point is that one would expect Cyt-*c* adsorption on the 4 μm TiO₂ film to be 2 times faster in comparison to the 8 μm film. However, the $t_{1/2}$ obtained is the same (~ 1 day) for both films. This may indicate that the diffusion of the protein molecules is not limited by the thickness of the TiO₂ films but by the movement of the protein molecules from the large pore channels to the smaller pores of the TiO₂ film.

3.3.2.3 Langmuir Isotherm

Previously, adsorption of flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD) and riboflavin (RF) on TiO₂ powder were found to obey the Langmuir isotherm (Shinohara et al., 1991). Also, the adsorption of the ruthenium dye N3 and the TCPP dye on nanocrystalline TiO₂ films gave a good fit to the Langmuir isotherm (Fillinger et al., 1999 and Cherian et al., 2000). In order to obtain a more fundamental understanding of the binding of Cyt-*c* and Hb on nanoporous TiO₂ films, their adsorption isotherms need to be determined.

In order to investigate the adsorption isotherms of Cyt-*c* and Hb onto nanoporous TiO₂ films, the quantity of each adsorbed protein was monitored as a function of protein concentration in solution. Data were collected following equilibration, which was reached after about 12 days for all protein concentrations.

Figures 3.14a and 3.14b show the adsorption isotherms for Fe(III)Cyt-*c* and MetHb adsorption to the TiO₂ films at pH 8. Both proteins are strongly adsorbed and stable at this pH as confirmed by their absorption maxima.

The experimental data were fit to a Langmuir isotherm. The straight lines shown in figures 3.14a and 3.14b result from fitting the data to the Langmuir adsorption isotherm (Shinohara et al., 1991).

$$K = \frac{\theta}{c(1-\theta)} = \frac{OD}{c(OD_{\max} - OD)}$$

Where K is the equilibrium binding constant, θ is the fractional occupation of adsorption sites, c is Cyt-*c* or Hb concentration in solution at equilibrium, OD the optical density of adsorbed Cyt-*c* or Hb (monitored at 406 nm) and $OD_{\max}$ is the maximum optical density observed at saturating protein concentrations. A good fit is

obtained to the Langmuir isotherm for both proteins, indicating the adsorption process is non-cooperative. From these fits, the binding constants (K_D) for Cyt-c and Hb were calculated to be $10.6 \times 10^4 \text{ M}^{-1}$ and $17 \times 10^4 \text{ M}^{-1}$ respectively.

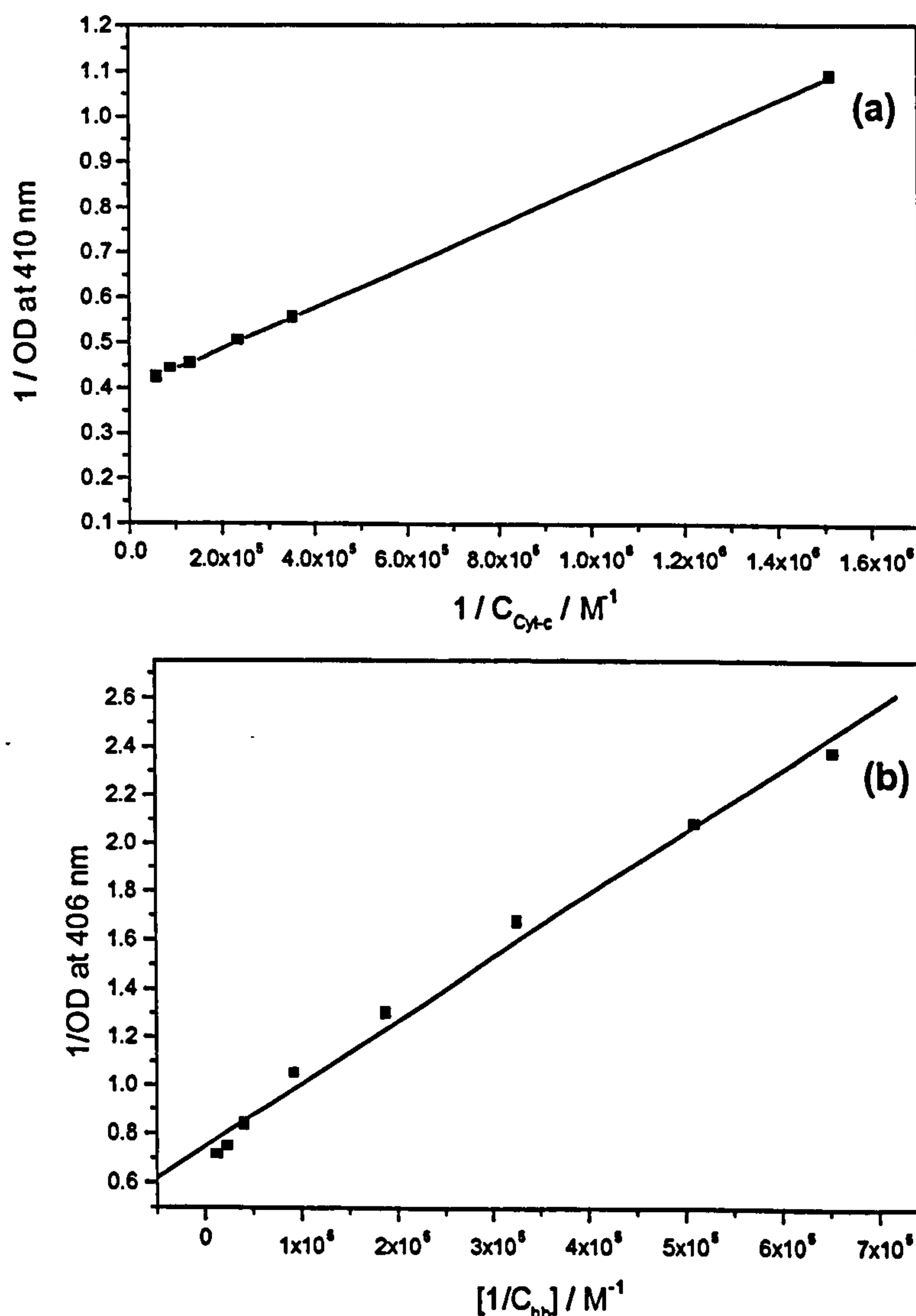
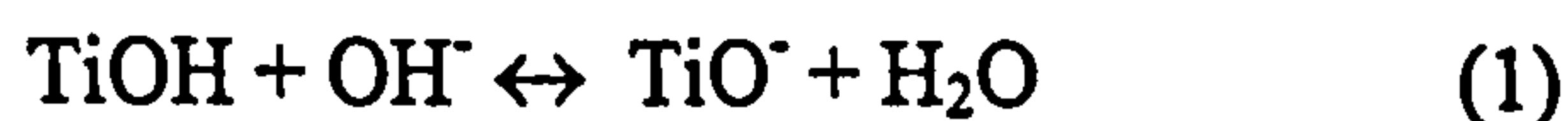


Figure 3.14: Adsorption isotherms of (a) Cyt-c and (b) Hb on nanoporous TiO₂ films at pH 7. Different Cyt-c and Hb concentrations were prepared using a 10 mM NaH₂PO₄ buffer solution of pH 7. The films were kept in the protein solutions for 30 days. $C_{\text{Cyt-c}}$ and C_{Hb} are the concentrations of Cyt-c and Hb left in each immobilising solution respectively after maximum protein adsorption was achieved. For each figure, the solid line is a best fit to a Langmuir adsorption isotherm, yielding a binding constant (K_D) of $10.6 \times 10^4 \text{ M}^{-1}$ and $17 \times 10^4 \text{ M}^{-1}$ for Cyt-c and Hb respectively.

3.4 Parameters Influencing Protein Adsorption

The pH and the salt concentration are known to have a strong influence on the surface charge of the TiO₂ films. Therefore they were chosen among other parameters such as the protein surface charge, the protein concentration, and the preparation time to examine the influence they have on the binding of proteins on the films.

The surface of the TiO₂ films will hydrolyse in the presence of water to form hydroxide layers at the surface (Ti-OH) (Lausmaa et al., 1998). The polar hydroxyl groups may cause the TiO₂ surface to attract and physically adsorb a single or several layers of polar water molecules. The surface ionisation of the hydroxyl groups on TiO₂ films is well represented as a function of pH by the following two equations (Mange et al., 1993 and Giacomelli et al., 1997, Rodriguez et al., 1996):



Where TiOH, TiO⁻ and TiOH₂⁺ are surface groups on the particles of the TiO₂ electrodes. At alkaline pHs (pH > 6.5) titanium hydroxide surfaces lose protons to produce negatively charged surfaces (Ti-O⁻) and at acidic pHs (pH < 6.5) adsorb protons to produce positively charged surfaces (Ti-OH₂⁺), which give an overall negative and positive charge to the TiO₂ surface respectively. The number of these sites and the surface charge of the titanium oxide particles are determined by the pH of the solution.

It is also known that Na⁺ ions adsorb electrostatically on the surface of the nanoporous TiO₂ films (Mange et al., 1993, Giacomelli et al., 1997, Janusz et al., 1997). Therefore, they neutralise (at least part of) the negative surface charge of the TiO₂ films,

a phenomenon known as complexation of the surface sites or electrostatic screening (see equation 3):



Na^+ ions bind strongly on the TiO_2 surface and they show very low mobility after binding.

3.4.1 Results

3.4.1.1 Effect of pH

The adsorption of redox proteins on TiO_2 films was expected to depend on the surface charge of the films, as has been seen for adsorption of proteins onto films of polyions (Ladam et al., 2001). Previous studies of the surface potential of the TiO_2 anatase aqueous solution interface indicate that the isoelectric point lies in the region of 6.1 - 6.3 (Shinohara et al., 1991). Therefore, the isoelectric point of the anatase TiO_2 film surface used in this project was assumed to be in between this range. This means that above $\sim$ pH 6.2 the TiO_2 surface is negatively charged. *Cyt-c* and Hb have isoelectric points of 10.5 and 7.5 respectively, meaning that *Cyt-c* and Hb are positively charged below pH 10.5 and 7.5 respectively (Collinson et al., 1992 and Antonini et. al., 1981).

Figures 3.15a and 3.15b show the saturation adsorption values for each of the two proteins binding on TiO_2 films at different pHs. Figure 3.15a shows a maximum amount of Cyt-*c* bound at pH 7 remaining fairly constant up to pH 10. Figure 3.15b shows that Hb binding is maximal between pH 7 and 8 and that it drops off out of this range.

When a TiO_2 film is immersed into protein solution of Cyt-*c* or Hb of e.g. pH 7, protons are released by surface hydroxyl groups giving rise into negative sites Ti-O^- . The higher the pH the more O^- groups are available. At pHs 7 to 10 Cyt-*c* remains positively charged and therefore a strong electrostatic interaction is expected. Hb is negatively charged at pHs > 7.2 and therefore beyond this point a lesser degree of electrostatic attraction is expected. At low pH ($\text{pH} < 6$) both proteins and the TiO_2 film are positively charged according to their IEP and therefore electrostatic attraction is weakened. Below pH 4.5 Cyt-*c* or Hb adsorption was undetectable and both proteins were partially denatured. These results support the importance of electrostatic interactions during protein binding.

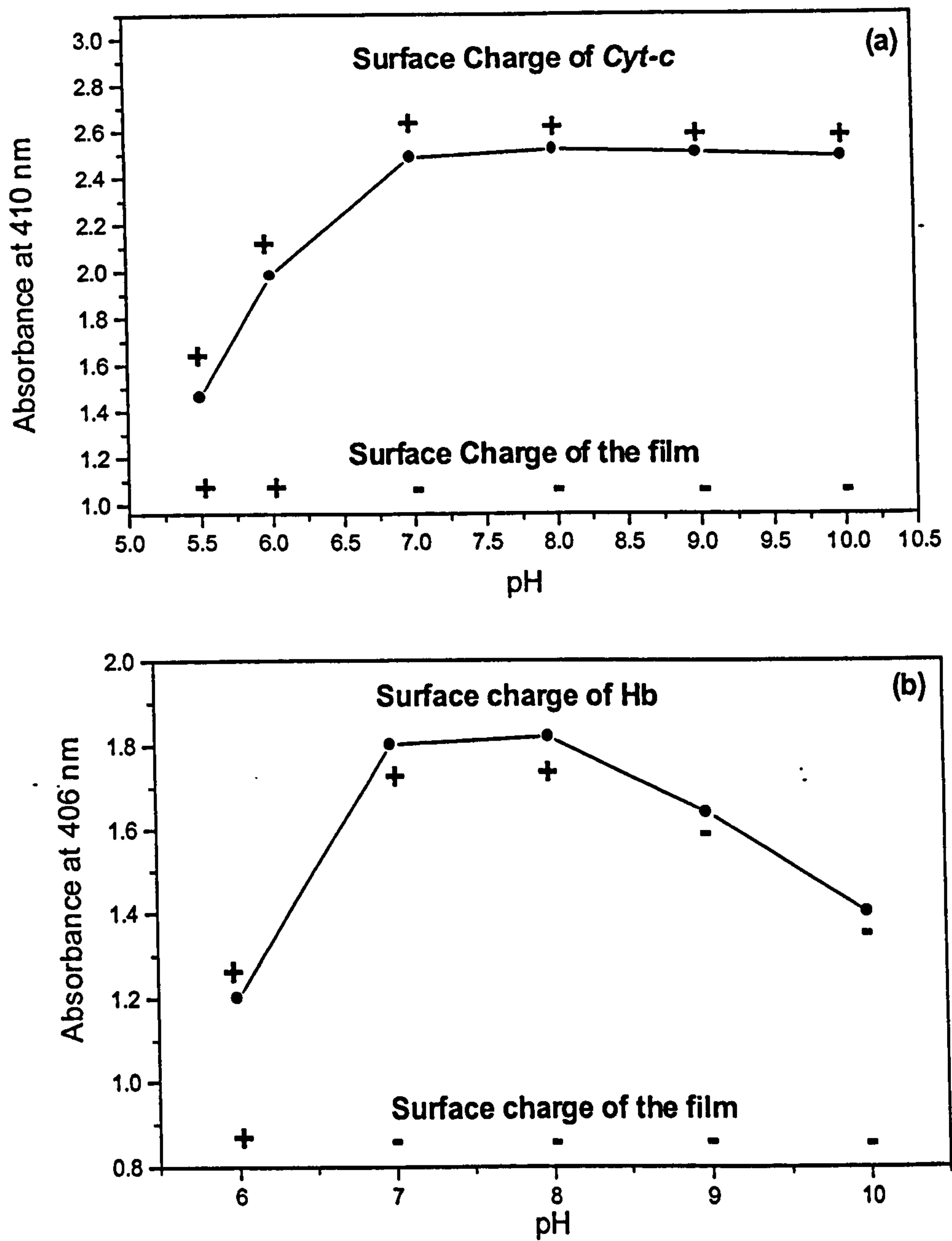


Figure 3.15: Effect of pH on the amount of (a) Cyt-*c* (b) Hb bound to a nanoporous TiO₂ film. Protein concentration for Cyt-*c* and Hb were 20 μ M and 18 μ M respectively and films were immersed in the solutions for 30 days. The overall charges of each protein and the TiO₂ film at each pH are also shown.

3.4.1.2 Dependence upon protein charge

In order to confirm further the dependence of protein adsorption on protein surface charge, a Cyt-*c* was purchased from Sigma, which had 60% of its lysine residues acetylated (Azzi et al., 1975). Lysines are positively charged and acetylation results in the loss of this charge. As a result the equilibrium adsorption constant, K_D , was an order of magnitude lower than that for native Cyt-*c* (Table 3.3). It is noted however that although less acetylated Cyt-*c* was adsorbed on to the TiO_2 film, it was still electrochemically active (see Chapter 4). This study further supports the importance of electrostatic interactions during protein adsorption.

Immobilised Protein	Adsorption constant K_D / M^{-1}	Desorption constant K_D / M^{-1}
Cyt- <i>c</i>	10.6×10^4	1.2×10^4
Acetylated Cyt- <i>c</i>	5.5×10^4	-
Hb	17×10^4	8.0×10^4

Table 3.3: Maximum Protein loading and protein binding constants (K_D) for native Cyt-*c* and *Acetylated* Cyt-*c* on 8 μm TiO_2 films.

3.4.1.3 Effect of ionic strength

In order to understand better the binding and the kinetics of protein adsorption on the TiO_2 surface, the effect of monovalent ions (NaCl) on the binding of Cyt-*c* molecules has been studied.

The adsorption constant K_D of Cyt-*c* on nanoporous TiO_2 films was determined for a range of NaCl concentrations in sodium phosphate buffer (0.01 M, pH 7). Figure 3.16 clearly shows that the binding of Cyt-*c* to the TiO_2 films depends to a great extent on the ionic strength of the solution from which the protein adsorbs. As ionic strength increases, significantly less protein is bound. The adsorption constant (K_D) falls from $6 \times 10^{-5} \text{ M}^{-1}$ in a NaCl free solution to $1 \times 10^{-5} \text{ M}^{-1}$ in 0.1 M solution of NaCl before falling close to zero at 0.5 M NaCl. It should be noted that Cyt-*c* becomes partially or completely denatured at NaCl concentrations higher than 0.3 M (Willit et al., 1987).

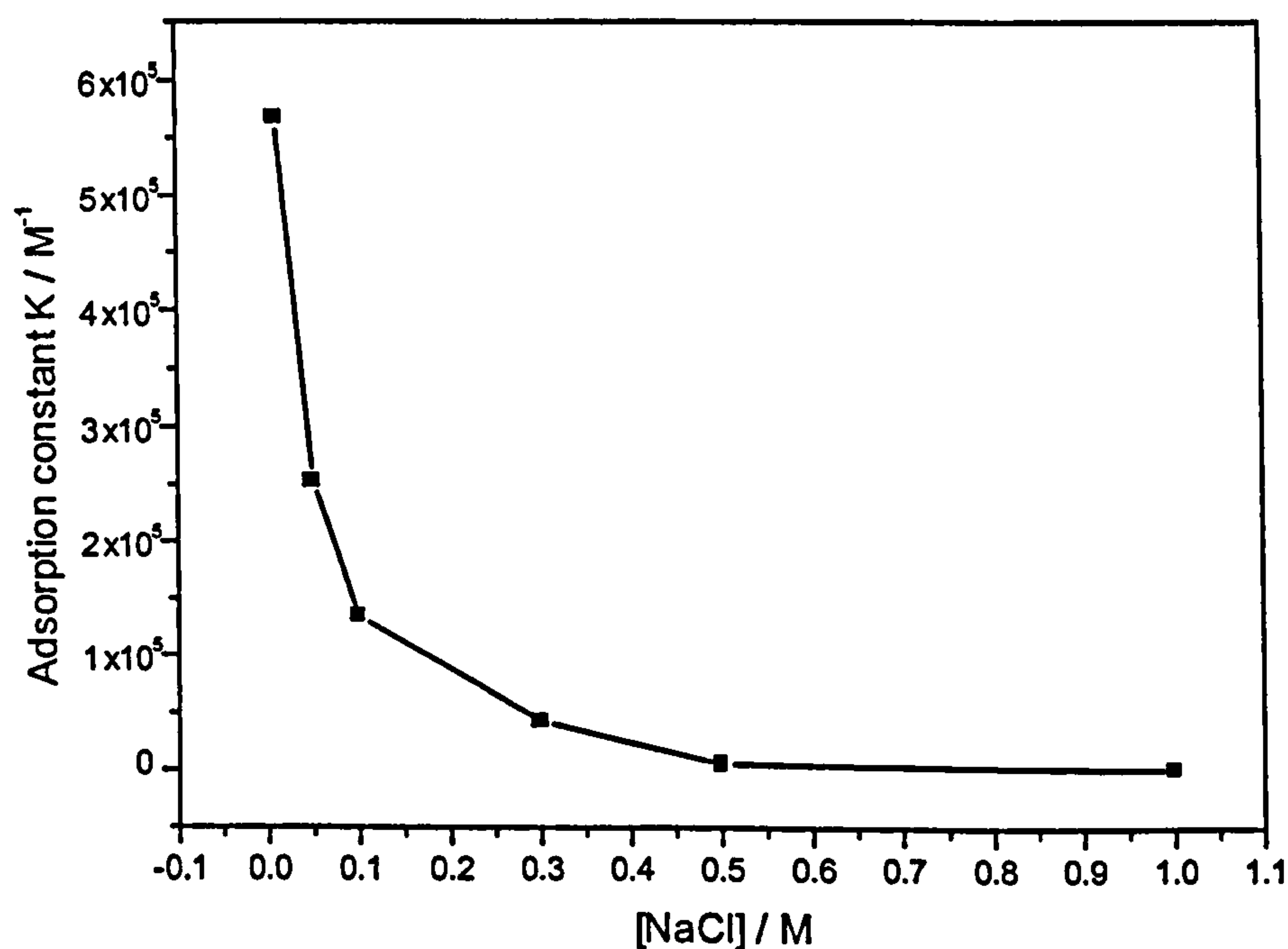


Figure 3.16: Effect of NaCl concentration on the binding strength of Cyt-*c* to TiO_2 . The binding constant (K_D) was calculated by fitting the adsorption to the Langmuir isotherm.

Finally, it is known that titanium dioxide has a very strong affinity for phosphate groups. Therefore, in order to find out if phosphate molecules from the buffer solution are competing with Cyt-*c* or Hb for adsorption sites on the films, a 10 mM Tris-HCl buffer solution of pH 8 was used instead. It was found that any possible binding of phosphate groups to the TiO₂ surface has no effect on the protein immobilisation process. The same protein (Cyt-*c* or Hb) immobilisation behaviour on the films was observed from both phosphate and Tris buffer solutions. In addition, incubation of immobilised Cyt-*c* or Hb TiO₂ films in phosphate buffer solutions for periods of up to several months did not result in any detectable degradation of the films.

3.4.1.4 Desorption in buffer solution (Irreversibility of binding)

Following adsorption of Cyt-*c* and Hb on nanoporous TiO₂ films (the film was kept in protein solution for three weeks), immobilised films were then immersed in protein free buffer solution in order to check the strength and reversibility of the binding.

Figure 3.17 shows that the immersion of Cyt-*c*/TiO₂ and Hb/TiO₂ films in protein free buffer solutions resulted in only 10 and 20 % desorption over 30 days respectively. The desorption was reasonably fast and reached a steady level after 10 days. The desorption constant K_D was $1.2 \times 10^4 \text{ M}^{-1}$ for Cyt-*c*, one order of magnitude smaller than the binding constants K_D of the protein (see Table 3.3). A more modest effect was observed for Hb with a binding constant of $1.7 \times 10^5 \text{ M}^{-1}$ and unbinding constant of $8 \times 10^4 \text{ M}^{-1}$.

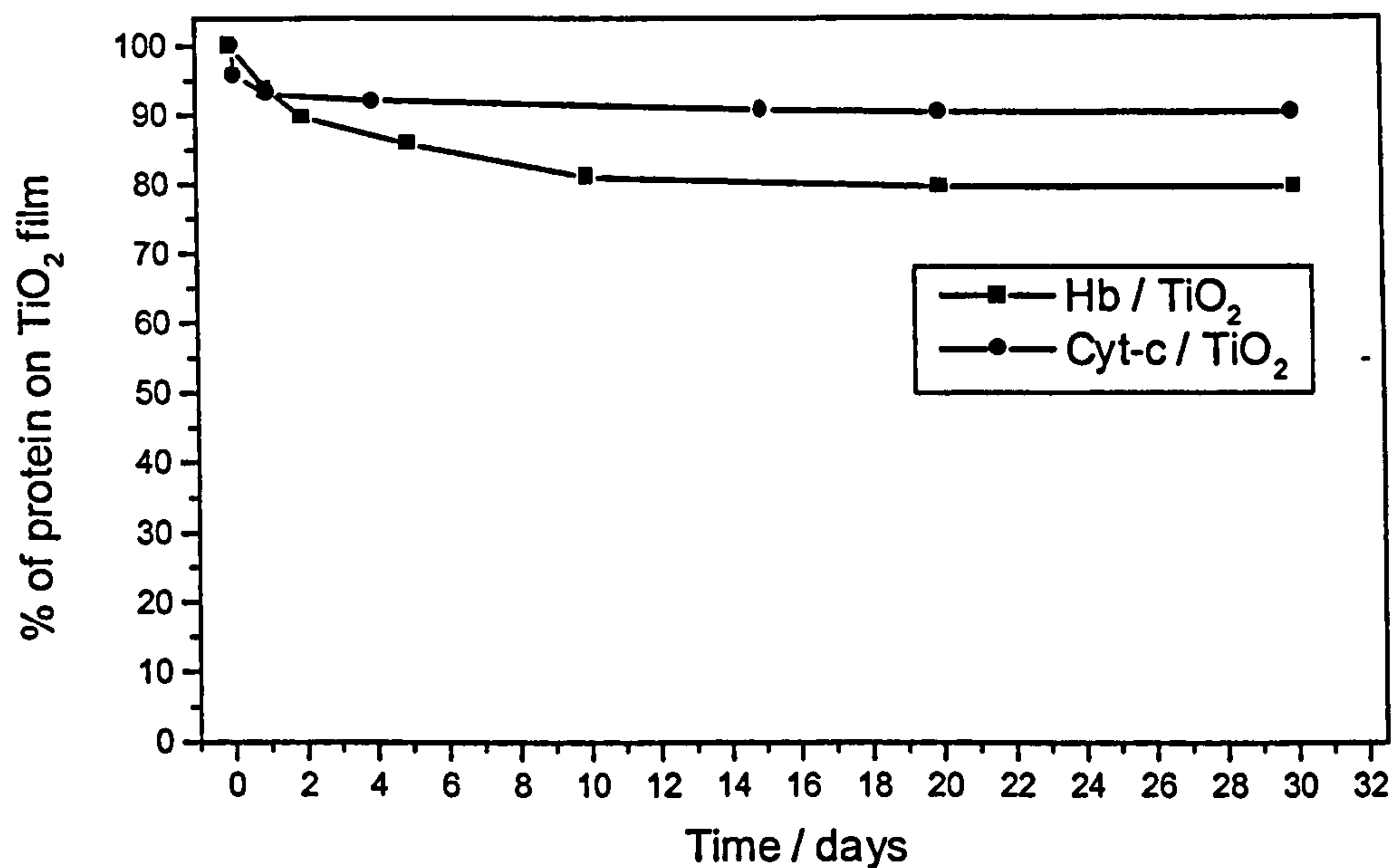


Figure 3.17: Cyt-*c* and Hb desorption from the nanoporous TiO₂ films into fresh protein free 4 ml of 10 mM NaH₂PO₄ buffer solution of pH 8 as a function of time.

After binding, both proteins are remarkably resistant to subsequent desorption, suggesting that protein binding to TiO₂ films is not entirely reversible. The K_D measured for the desorption process is different from the K_D for the adsorption process, bringing the assumption of equilibrium conditions into question.

3.4.1.5 Effect of NaCl on desorption

The effects of NaCl ions on the desorption of Cyt-*c* from the TiO₂ films were also examined. Figure 3.18 shows that the incorporation of a 10, 100 and 500 mM NaCl concentration in protein free buffer solutions of pH 7, where Cyt-*c*/TiO₂ films were immersed after immobilisation, resulted in 15, 65 and 90% desorption of immobilised Cyt-*c* respectively.

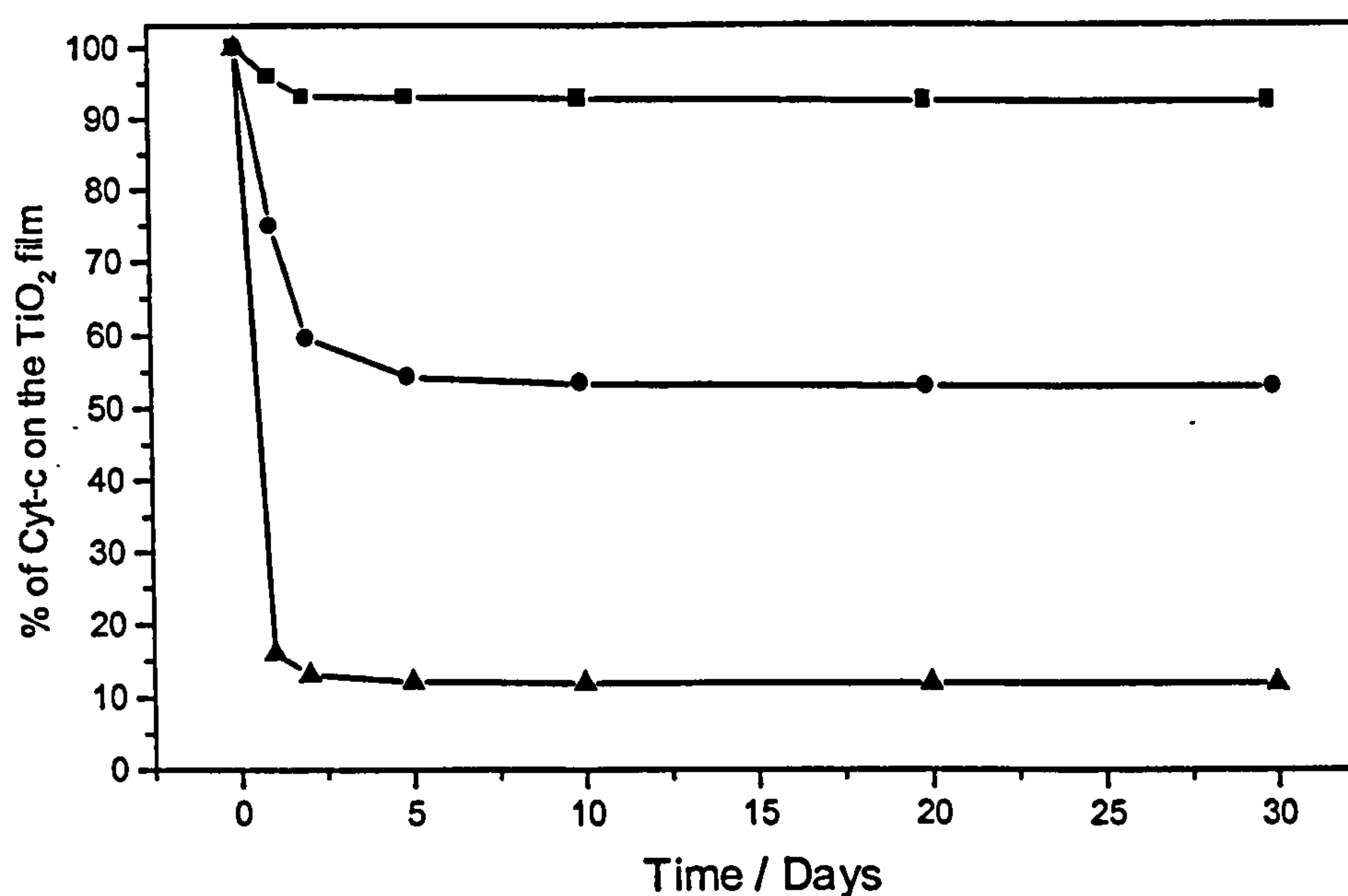


Figure 3.18: Effect of: 0.01 (■), 0.1 (●) and 0.5 M (▲) NaCl on the desorption of Cyt-*c* from TiO₂ films.

The higher the amount of NaCl added the higher the amount of protein desorbed from the film. Therefore, it is possible to overcome the protein resistance to desorption by adding high concentrations of NaCl to the buffer, with near complete desorption at 0.5 M NaCl.

3.4.1.6 Effect of time on desorption

Finally, the effect of the immobilisation period of the TiO_2 films on the desorption of Cyt-c from the film was also examined. Two Cyt-c/ TiO_2 films were prepared with the same amount of protein on the surface. The only difference between the two films was that one had spent 8 days in protein solution and the other 30 days. Figure 3.19 shows the desorption kinetics of both films in a protein free phosphate buffer solution containing 0.1 M NaCl. The desorption process was monitored over a period of 30 days. UV/Vis spectroscopy showed that 70 % of the protein desorbed from the film that had spent 8 days in protein solution but only 50 % desorbed from the film that had spent 30 days in protein solution. The 20% difference in desorption indicates that the protein becomes more strongly bound to the film when left longer in the protein solution.

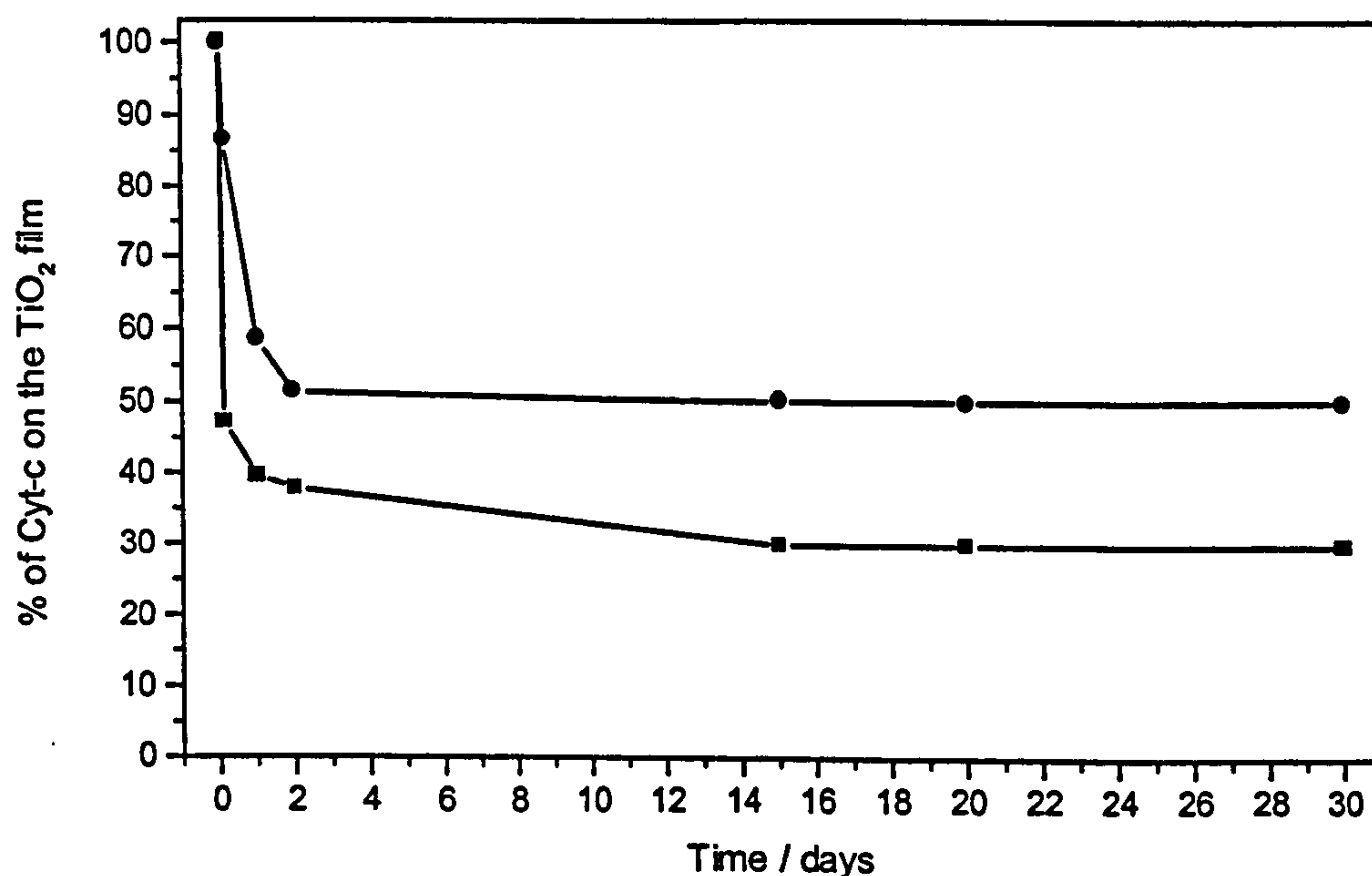


Figure 3.19: Effect of the immobilisation time on protein desorption. Films were stored in 0.1 M sodium phosphate buffer of pH 7. Film that had been in Cyt-c solution for 8 days (■), film that had been in Cyt-c solution for 30 days (●).

These results indicate that there are two Cyt-*c* populations adsorbed on the film, one which is easy and one which is harder to desorb.

3.4.2 Discussion

Effect of pH: It was found that pH has a significant effect on the binding of proteins on the TiO₂ films. This is most probably the result of the influence of pH on both the overall charge of the protein and the overall charge of the TiO₂ surface. The adsorption of Cyt-*c* and Hb on the nanoporous TiO₂ films is optimum for solution pH at which the film and the protein electrostatic charges were counterpoised, consistent with the adsorption process being significantly controlled by electrostatic interactions between the film surface and the protein. In other words for the optimum electrostatic interaction the respective charges must be opposed (Topoglidis et al., 2001).

However, there is still significant binding of both Cyt-*c* and Hb in conditions where they carry the same overall charge as the TiO₂ film. Although this appears counter intuitive, it is in accordance with other findings that proteins can bind to layers of polyions regardless of their charge (Ladam et al., 2001). This is thought to happen because although the overall charge of the protein may be for example negative, it may still have positive patches on its surface which allow localised binding to a negatively charged surface.

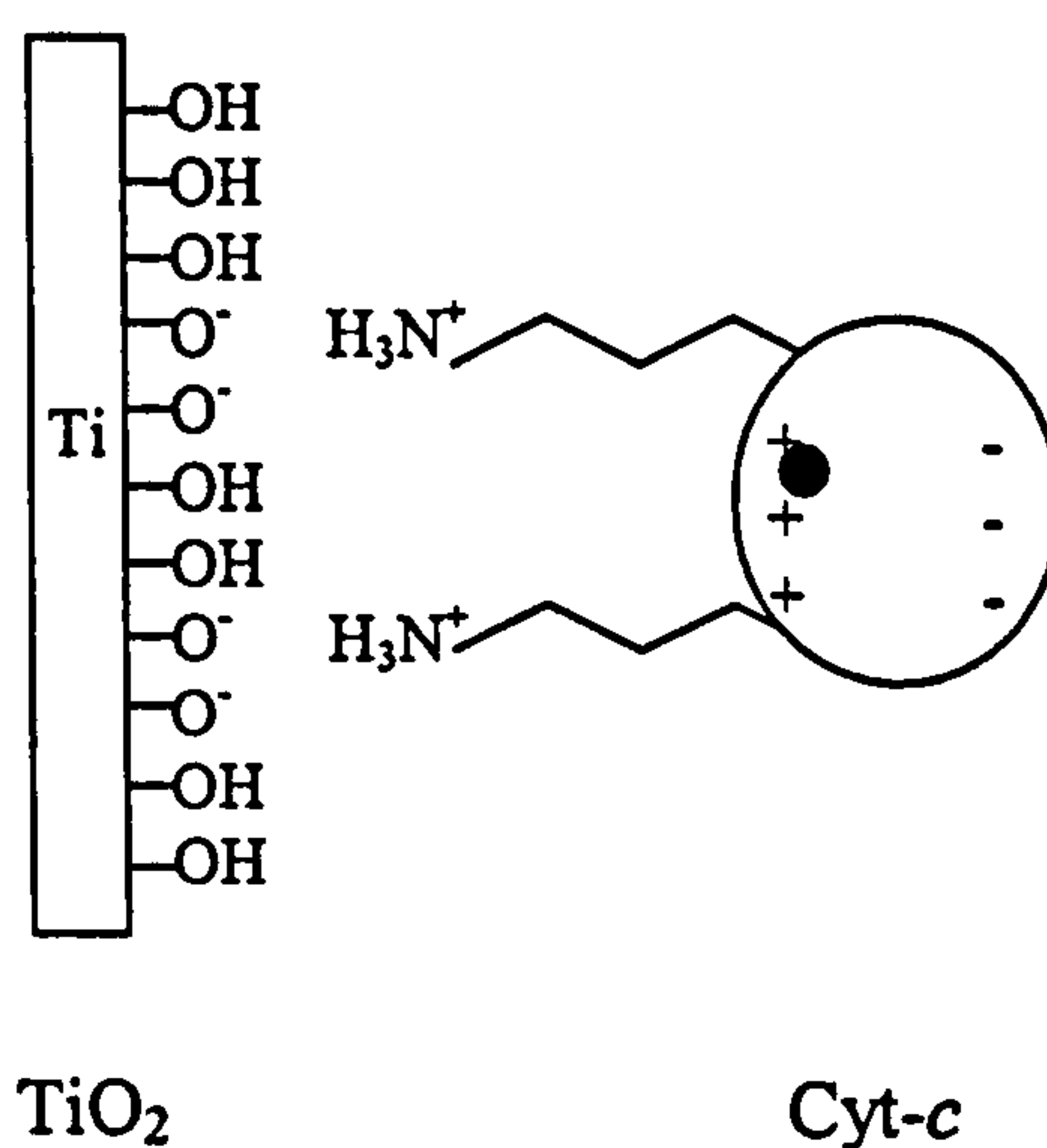
This adsorption pattern can be also explained from the distribution of the surface charges on the TiO₂ film at a particular pH. The TiO₂ nanoporous film has a range of crystal surfaces, which are expected to have different IEPs (Rodriguez et al., 1996),

resulting in an inhomogeneous charge distribution. At low pHs there are more OH_2^+ groups and at high pHs more O^- groups and there is always an equilibrium reached between the two at a certain pH. Therefore, even at pH 6 for Cyt-c and at pHs 6, 9 or 10 for Hb we could see strong electrostatic binding between the proteins and the film even if the electrostatic charges are not counterpoised. As the pH is increased ($\text{pH} > 7$), the TiO_2 surface charge becomes more negative and hence the electrostatic repulsion between TiO_2 and the protein carboxylate functionality is increased. On the other hand, at high pHs the COOH groups of the acidic amino acids on the protein become COO^- and they can bind on the Ti^{4+} ions on the TiO_2 surface. Therefore all these factors contribute to the protein binding also but they don't control it.

Catalase and cytochrome P450 are two heme proteins, which have low IEPs, of 5.4 and 4.8 respectively. Therefore, they are negatively charged at pHs above their IEP. The number of lysine residues present on these two proteins is also much less than on Hb and Cyt-c. The TiO_2 film is negatively charged above pH 6 and therefore it doesn't attract electrostatically catalase and cytochrome P450 in the pH region (6-10). Therefore, attempts to bind these two proteins by physical adsorption on the TiO_2 films were unsuccessful. These results are further evidence that the binding is electrostatic and that negatively charged proteins (i.e. with low IEP) do not bind on the TiO_2 films and an alternative way of binding needs to be developed. For example initially, a polylysine population could be adsorbed in the TiO_2 film before it is immersed in the negatively charged protein solution. Such a study is taking place at the moment in Dr. Durrant's group.

Effect of protein charge: The importance of the surface charge is further confirmed by the fact that on acetylation of 60% of the lysine residues, Cyt-*c* binds less strongly than in its native state. This fact is reflected by the equilibrium adsorption constant of acetylated Cyt-*c*, which is an order of magnitude lower than for native Cyt-*c* (Topoglidis et al., 2001).

The strong adsorption of Cyt-*c* on nanoporous TiO_2 films, is mainly (primarily) due to the electrostatic interactions of the positively charged amino acids (mainly lysines) of Cyt-*c* and the oxygen moieties of the negatively charged surface of the TiO_2 at pH 7 (see Scheme 3.1). The acetylation of the lysines of Cyt-*c* could specifically prevent lysines from binding on the surface of the TiO_2 film or more generally shift the IEP of Cyt-*c* to a value lower than 10. In both cases the result would be less Cyt-*c* binding on the TiO_2 films:



Scheme 3.1: Illustration of the mode of electrostatic interaction between the Lysine residues on Cyt-*c* ($-\text{NH}_3^+$) and the negatively charged oxygen moieties on the TiO_2 surface at pH 8. The schematic is for illustrative purpose only and is not drawn to scale.

Of particular note, a group of lysine residues (among which lysines: 13, 27, 72 and 79) is centred around the heme edge of Cyt-*c* and therefore it is expected to orient the exposed heme edge of the protein facing the negatively charged surface of the film. These lysine residues of Cyt-*c* are known to be central to the recognition and binding of cytochrome reductase and oxidase, which are negatively charged. This orientation of Cyt-*c* on the TiO₂ film should therefore favour electron transfer between the TiO₂ film and the adsorbed Cyt-*c* (detailed discussion of this issue follows in Chapter 4).

Effect of ionic strength: Investigation of the effects of salt concentration on binding of Cyt-*c* shows another interesting aspect of this system. Increasing the salt concentration of the Cyt-*c* immobilising solutions, leads to a lower adsorption constant K_D indicating weakened binding between the protein and the TiO₂ films (Topoglidis et al., 2001). This is probably the result of increased screening of the TiO₂ surface by the sodium ions, weakening the strength of the electrostatic interaction. Na⁺ ions adsorb electrostatically on the surface of the nanoporous TiO₂ films and they show very low mobility after binding. Therefore, they neutralise (at least part of) the negative surface charge of the TiO₂, a phenomenon known as electrostatic screening and it is difficult for the Cyt-*c* molecules to replace them (Janusz et al., 1997 and Giacomelli et al., 1997). This accounts for the low protein binding in the presence of high NaCl concentration. This is also analogous to what one would expect while carrying out ion exchange chromatography and again reflects the electrostatic nature of the Cyt-*c*/TiO₂ interaction.

Desorption Studies: However, the desorption of protein from the nanoporous TiO_2 films does not wholly conform to a simple electrostatic model. If referring to simple electrostatic binding laws, the immobilised proteins should unbind as easily and efficiently as they bind on the TiO_2 films. However, this is not the case here as the amount of Cyt-*c* or Hb desorbed in protein free phosphate buffer solution is very low. This couldn't be a change in the kinetics of unbinding versus those of binding because even when the immobilised films were left in protein free buffer solution for a couple of months, no more Cyt-*c* or Hb were desorbed. The degree of desorption measured for Cyt-*c*/ TiO_2 and Hb/ TiO_2 films was much less than that expected on the basis of the K_D for adsorption, with K_D values for desorption being lower than for adsorption (Topoglidis et al., 2001).

In addition Cyt-*c*/ TiO_2 films that had been left for one month compared to eight days demonstrated significantly greater resistance to desorption (Topoglidis et al., 2001). This suggests that over this extended period when saturation of binding has occurred, the amount of protein on the surface does not significantly increase, the strength of the binding does. In other words the initial electrostatic attraction of protein to surface serves to put it in a position where it can find its optimum binding orientation. This additional binding strength might be the result of covalent binding of carboxylate residues to Ti^{4+} sites or simply orientation of the protein to have the maximum amount of positive charge in contact with the negatively charged TiO_2 surface. This is analogous to the model used to explain binding of tetracarboxylic organic dyes to TiO_2 films in which binding was also seen to lower K_D for desorption with respect to that of adsorption. In this case initial fast interactions are attributed to the ligation of one carboxylate moiety to a Ti^{4+} binding site (Fillinger et al., 1999). This is then followed by a slower step involving the alignment and binding of another carboxylate moiety and a

Ti⁴⁺ binding site. This type of binding makes desorption less likely since the chance of both carboxylates being dissociated from the TiO₂ surface at the same time is low.

3.5 Conclusion

A detailed study of the binding of proteins on the nanoporous TiO₂ films was attempted in this chapter. Protein immobilisation on the TiO₂ films is found to be remarkably stable, it obeys the Langmuir isotherm and the time required for saturation of binding is roughly dependent on diffusion of the protein through the film. Among the factors having an important role in influencing the binding, the pH, the protein surface charge, the ionic strength of solution, the protein size and the immobilisation time were identified. Primarily the binding is probably electrostatic involving the attraction of the lysine residues on the surface of the protein to the O⁻ moieties on the surface of the TiO₂ film. However, the stability of the binding is attributed to secondary binding processes occurring after the initial immobilisation. These processes could be covalent binding of carboxylate residues to Ti⁴⁺ sites of the TiO₂ film or simply orientation of the protein to have the maximum amount of positive charge in contact with the negatively charged TiO₂ surface.

The high and strong protein adsorption achieved is an advantage for the development of biosensors or for studying the electron transfer reactions between the adsorbed protein and the TiO₂ electrode.

CHAPTER 4

SPECTROELECTROCHEMISTRY AND ELECTROCHEMISTRY OF HEME PROTEINS IMMOBILISED ON NANOPOROUS TiO₂ FILMS

4.1 Introduction

4.1.1 Electrochemistry of proteins on electrodes

Electron transfer reactions have been studied electrochemically between a wide range of redox proteins and electrode surfaces, in which the protein/electrode electron transfer is monitored as a current in an external circuit (Bowden et al., 1997, Taniguchi et al., 1997, Dong et al., 1997, Katz et al., 1997, Hill et al., 1996, Armstrong et al., 2000a). Such electron transfer requires electrical contact between the protein's redox center and the electrode's interface (Yang et al., 1999, Taniguchi et al., 1992, Cooper et al., 1993, Heller et al., 1992). In many instances direct and reversible electron transfer between the adsorbed protein and the electrode is inhibited due to the insulation of the active site (heme) by the protein matrix. Typically, the protein matrix separates the redox-site from the electrode surface and practically insulates the active center from electrical contact with electrode interfaces. Other factors which do not favour electron transfer include denaturation of the protein due to adsorption on the electrode, unfavourable orientation of the protein on the electrode, low protein loading and adsorption of other small molecules on the electrodes (Huang et al., 1996, Armstrong et al., 2000a).

Diffusional electron mediators as charge transporters and promoter molecules attached on the surface of the electrode, have been employed in order to optimise the transfer of electrons between the active redox center of the protein and the electrode surface (Katz et al., 1997, Ye et al., 1988, Hill et al., 1993, Schlereth et al., 1992). At these modified electrodes rapid electron transfer can take place.

4.1.2 TiO₂ Electrodes

N-type nanocrystalline semiconductors based on metal oxide materials, like TiO₂, have attracted considerable attention because of their unusual electrochemical and photochemical properties and the convenience with which they can be assembled in high area thin film or colloidal form.

Nanoporous, nanocrystalline TiO₂ films are wide band gap semiconductors (~3.2 eV) and therefore optically transparent (Hagfeldt et al., 1995). They are constituted by a network of mesoscopic particles, which are interconnected to allow for electronic conduction to take place. For these semiconductors, reactive electrons can be supplied electrochemically by externally biasing at potentials more negative than the conduction band edge (Rothenberger et al., 1992). They also can be generated photochemically, either by band gap excitation and electron/hole pair generation, followed by hole scavenging (Bahnemann et al., 1984), or by electron injection from sensitizer dyes (Tachibana et al., 1996, Durrant et al., 1999). Several studies of metal oxide semiconductors in nanocrystalline form have shown that electron addition via both schemes can be observed optically. However, the relatively slow electron mobility within the nanocrystalline TiO₂ at low negative potentials precludes the use of cyclic voltammetry to determine dye/electrode electron transfer kinetics (Boschloo et al., 2000, Rothenberger et al., 1992).

4.1.3 Spectroelectrochemistry: Generation of Ti^{3+} States

TiO_2 films like other metal oxide semiconductors interact with H^+ and OH^- ions as shown in equation 1 (Mange et al., 1993).



Nanocrystalline TiO_2 films are optically transparent to visible light due to their wide-band gap (3.2 eV). The application of negative potentials to the nanocrystalline TiO_2 film results the black coloration of the film and in a broad absorption increase due to the formation of Ti^{3+} ions, attributed to optical absorption of electrons occupying conduction band/intraband states of the film (Rothenberger et al., 1992, Boschloo et al., 1999a, 1999b, 2000). Monitoring of this absorption increase as a function of applied potential is therefore a crude measure of the occupancy of these states, and therefore the electronic density of states of the film.

4.1.4 Aims

In this chapter the ability of Cyt-c and Hb to undergo redox reactions after adsorption on nanoporous TiO_2 films will be monitored by optical spectroscopy (spectroelectrochemically) and by cyclic voltammetry, with the protein/ TiO_2 electrode comprising the working electrode of a three electrode electrochemical cell. The same techniques would be also used to examine the electrochemical properties of these films

and how the wide band gap of titania (3.2 eV) affects their utility as working electrodes. Finally the electrochemical behaviour of Cyt-c is studied on another metal oxide, nanocrystalline ZnO films, and a comparison between the two n type metal oxides is attempted.

4.2 Experimental Methods

4.2.1 Protein Immobilisation

Immobilisation of Cyt-c and Hb on the nanoporous TiO₂ films and of Cyt-c on the nanocrystalline ZnO films was achieved as described in chapters 2 and 3. All films were left in protein solution for at least 2 weeks before use. Prior to all (spectro)electrochemical measurements, films were removed out from the immobilisation solutions and rinsed with buffer to remove non immobilised protein.

4.2.2 Spectroelectrochemical Studies

Electrochemical measurements were conducted in the homebuilt 3 electrode electrochemical cell (see chapter 2) employing a blank or with protein immobilised TiO₂ or ZnO film as the working electrode, a platinum mesh as counter electrode and an Ag/AgCl electrode (saturated KCl) as reference electrode. The above cell was placed in

a Shimadzu UV-1601 spectrophotometer and potential control was provided by an Autolab PGstat 12 computer controlled potentiostat. The absorption changes of the blank or with protein immobilised TiO_2 or ZnO films were monitored as a function of the applied potential. Contributions to the spectra from scatter and absorption by the TiO_2 or the ZnO film alone were subtracted by the use of protein free reference films. The electrolyte solution used was 2.5-3.5 ml of 10 mM NaH_2PO_4 at pH 7, which was degassed with argon prior to and during any optical / electrochemical measurements unless otherwise specified. All experiments were carried out at room temperature and all potentials quoted with respect to Ag/AgCl (0 V vs Ag/AgCl being equivalent to ~ 0.222 V vs NHE).

4.2.3 Cyclic Voltammetry

Cyclic voltammetry on blank or immobilised with protein TiO_2 or ZnO films was carried out over the potential range -0.7 to +0.1 V vs the Ag/AgCl electrode at a scan rate of 0.1 V/s and at a step potential of 0.00488 V unless otherwise stated. Potential control was provided by an Autolab PGstat 12 computer controlled potentiostat. All experiments were carried out at room temperature.

4.3 Results

4.3.1 Electrochemical activity

Having immobilised Cyt-*c* and haemoglobin on nanoporous TiO₂ films successfully (see Chapter 3), the ability of these redox proteins to retain their redox activity after immobilisation on the TiO₂ electrodes was examined. This was done by either adding redox mediators to a cuvette containing the protein/TiO₂ electrodes or electrochemically by the application of negative or positive potentials without the addition of any redox mediators or promoters. The redox state of the heme was determined spectroscopically by UV/Vis absorption spectra.

It was found that the immobilised Cyt-*c* or Hb on the TiO₂ electrodes could be readily cycled between their Fe(II) and Fe(III) states by respectively the addition of sodium dithionite and bubbling the buffer solution with oxygen (Topoglidis et al., 1998).

Alternatively, an electrochemical study of Cyt-*c* and Hb was undertaken to study their redox behaviour and properties after immobilisation on the TiO₂ films. Cyt-*c* could be readily reduced electrochemically by the incorporation of the Cyt-*c*/TiO₂ film as the working electrode of the 3 electrode electrochemical cell. The application of a negative potential (≤ -0.3 V vs Ag/AgCl) to the conducting glass substrate of the Cyt-*c*/TiO₂ electrode resulted in the reduction of Fe(III) Cyt-*c* to Fe(II) without the addition of any redox promoters or mediators, as illustrated in figure 4.1a (Topoglidis et al., 1998, 2000).

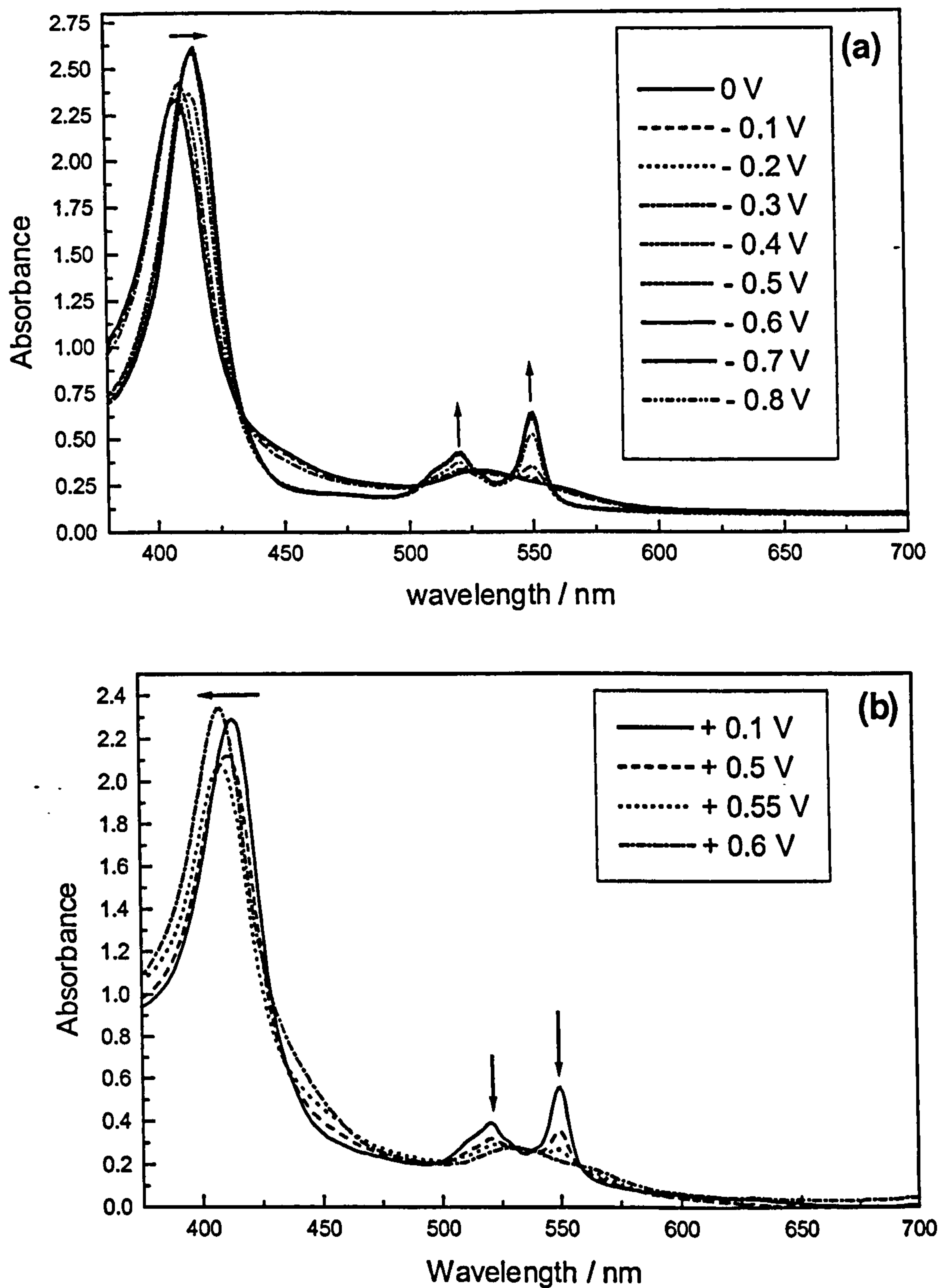


Figure 4.1: UV-Vis spectral changes for the (a) electrochemical reduction of the oxidised Fe(III) Cyt-c/TiO₂ under increasing negative potentials (0 to -0.8 V) and (b) the electrochemical oxidation of Fe(II) Cyt-c/TiO₂ under increasing positive potentials (0 to +0.6 V).

The sharp α and β bands at 550 and 520 nm of ferrocytochrome c (Fe(II)Cyt-c) increased as the immobilised Cyt-c was progressively reduced by applying increasingly negative potentials. Simultaneously, the Soret band at 410 nm was shifted to 416 nm. The absorption spectrum of the electrochemically reduced Fe(II) Cyt-c/TiO₂ film shows the characteristic Fe(II) heme absorption bands at 416 and 520 and 550 nm and these bands are in good agreement with the solution spectrum of Cyt-c (Wood et al., 1984). The application of positive potentials (0 to 0.6 V) to the electrode and/or the bubbling of the buffer solution with oxygen, resulted in the re-oxidation of the immobilised Cyt-c (see Figure 4.1b).

The ability of immobilised Hb to retain its redox activity electrochemically after its adsorption on the TiO₂ film was also tested. UV-Vis spectra of the immobilised Met Fe(III)Hb on the TiO₂ film were monitored after 15 minutes at the given negative potential of (0 to -0.7 V). According to figure 4.2a, the Soret band at 406 nm of the immobilised oxidised Met Fe(III)Hb decreased, a new peak at 428 nm for the reduced deoxy Fe(II)Hb increased gradually and another peak at 558 nm was also formed. These spectral changes correspond to the absorption spectrum of deoxy Fe(II)Hb in solution (Antonini et al., 1971) and show that the immobilised Hb was electrochemically reduced under negative bias without the addition of any promoters or mediators (Topoglidis et al., 2000).

Similarly, by applying positive potentials (0 to 0.6 V) vs Ag/AgCl and by bubbling argon at the same time, the re-oxidation of the immobilised deoxy Fe(II)Hb was achieved. According to figure 4.2b the Soret band at 428 nm decreased and the band at 558 nm of the deoxyHb gradually disappeared and instead the band of 406 nm of Met Fe(III)Hb started increasing again confirming that the immobilised Hb was re-oxidised successfully.

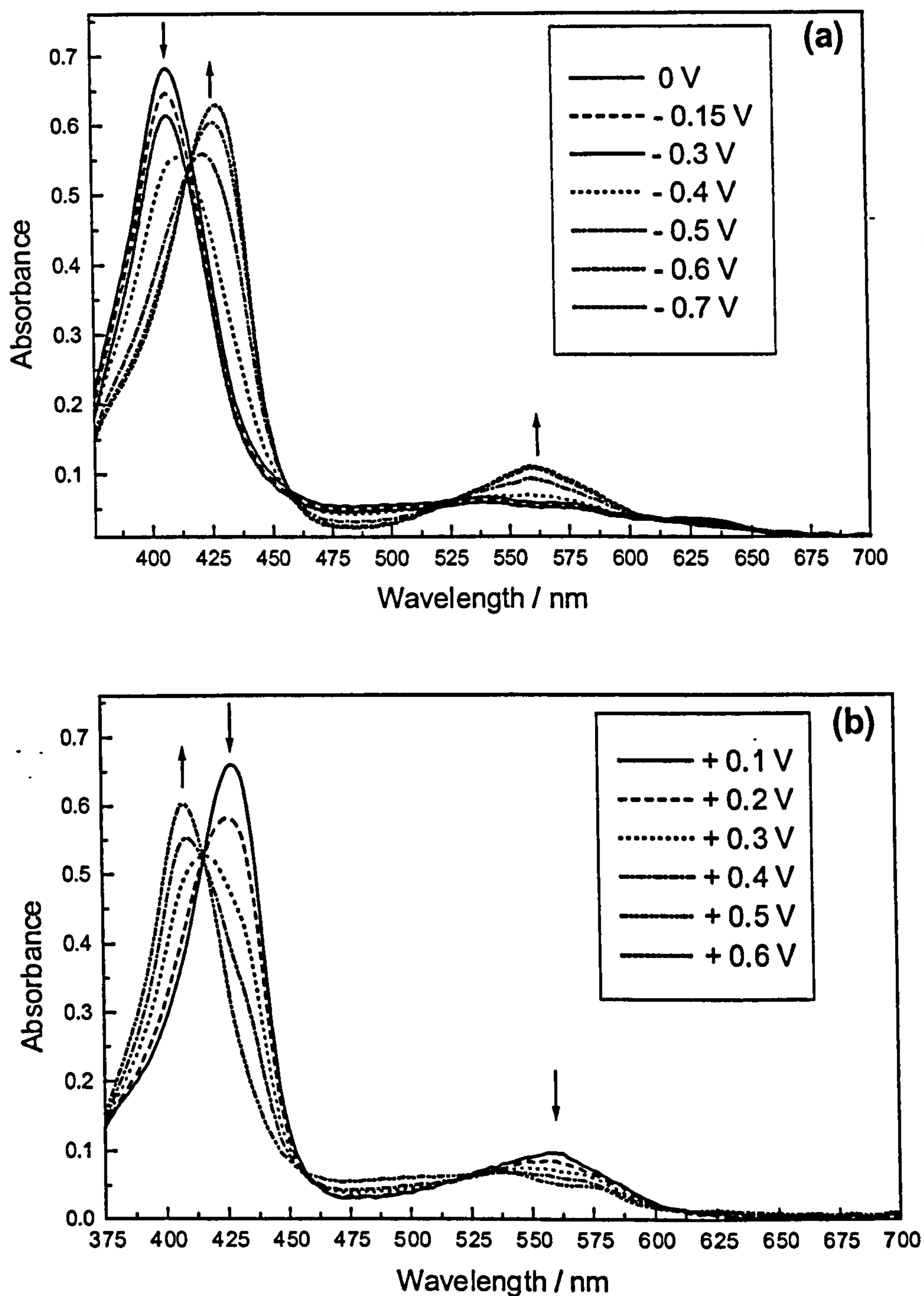


Figure 4.2: UV-Vis spectral changes for the (a) electrochemical reduction of Met Fe(III)Hb/TiO₂ under increasing negative potentials (0 to -0.7 V) and (b) the electrochemical oxidation of deoxy Fe(II)Hb/TiO₂ under increasing positive potentials (0 to +0.6 V).

4.3.2 Kinetics

Figures 4.3a and 4.3b show the time dependent absorbance changes at 550 and 428 nm for Cyt-c/TiO₂ and Hb/TiO₂ respectively. These absorption increases are associated with the reduction of Cyt-c and Hb. A potential dependence of the rate of reduction of each protein and also on the extent to which each protein became reduced was found. The kinetics of reduction are relatively slow for both proteins. At lower negative potentials (-0.3 to -0.6 V) not all of the immobilised Cyt-c or Hb gets reduced. The absorption reduction peaks increase but they never reach their maximum value (indication of complete protein reduction) but saturate at a lower value. Prolonged studies for both proteins showed that even after a couple of hours the immobilised proteins never reach complete reduction. For example at -0.4 V only 50% of Cyt-c was reduced and it took more than ten minutes to reach a steady level (even after 2 hours no more protein was reduced)

All of the immobilised Cyt-c or Hb gets reduced when the applied bias is ≥ -0.6 V and the reduction of each protein reached completion after 30 seconds. Hb was harder to reduce than Cyt-c, being only 10% reduced at -0.4 V and taking 90 seconds to be 95% reduced at -0.6 V. The kinetics of reduction for the different bias steps indicate that the limited (small) reduction observed at potentials lower to -0.6 V is not limited by these kinetics.

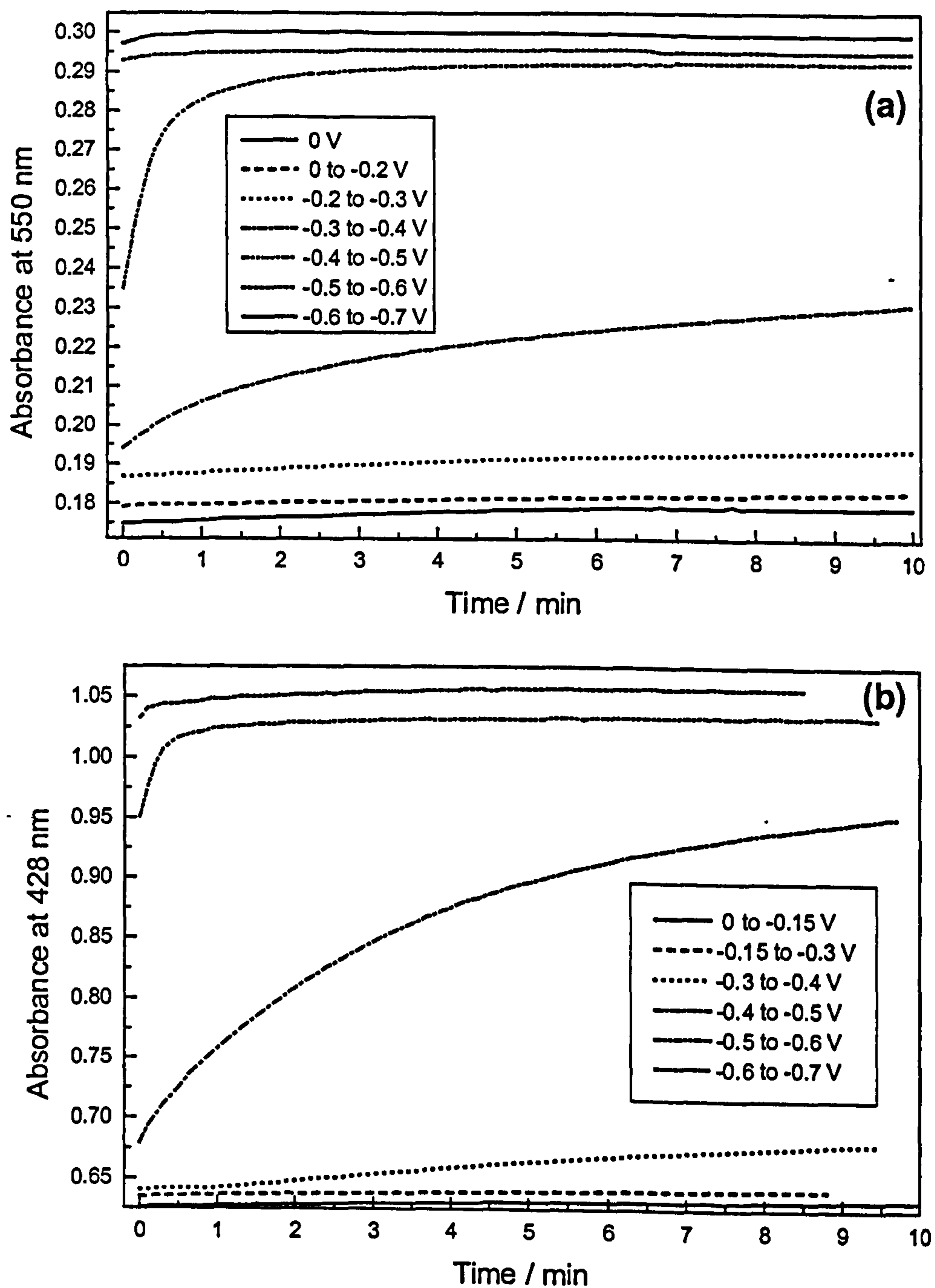


Figure 4.3: Time dependent absorbance changes (a) at 550 nm for Cyt-c/TiO₂ and (b) at 428 for Hb/TiO₂ showing the progressive reduction of each protein on the surface of the TiO₂ films, following incremental potential steps.

Figures 4.4a and 4.4b show the rate of reduction of Cyt-c at 550 nm and of Hb at 428 nm under the direct application of a $0 \rightarrow -0.7$ V potential step. The $t_{1/2}$ for the complete electrochemical reduction of the immobilised Cyt-c or Hb is approximately 10-15 seconds. This is the fastest time required for the electrochemical reduction of all the immobilised Cyt-c and Hb.

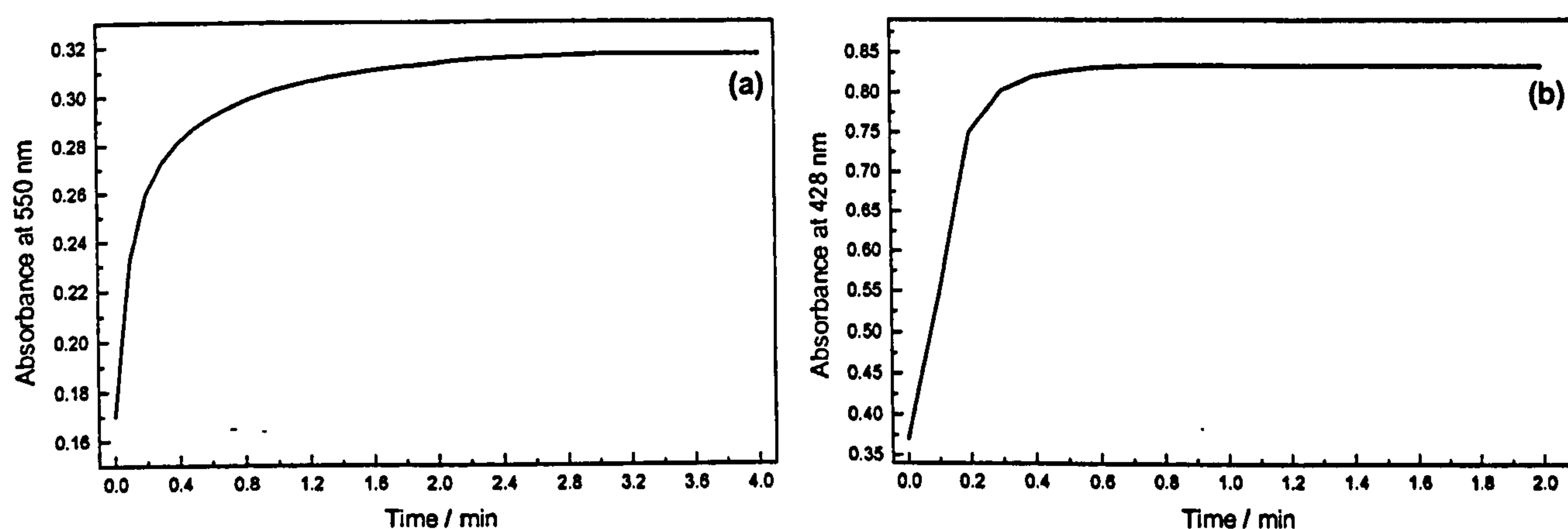


Figure 4.4: Rate of reduction of (a) Cyt-c/TiO₂ at 550 nm and (b) Hb/TiO₂ at 428 nm, under the application of $0 \rightarrow -0.7$ V potential step.

The re-oxidation kinetics for both immobilised proteins are much slower than the reduction kinetics. Due to the very low conductivity that the TiO₂ show at positive potentials, it takes 10-20 min for immobilised Cyt-c and Hb to get re-oxidised depending on the applied positive potentials. The slow re-oxidation of the immobilised proteins (Cyt-c and Hb) cannot be caused by residual oxygen in the buffer solution. The buffer solution is bubbled with Argon before and during use for a long period of time in order to establish inert conditions. Also in the case of immobilised Cyt-c, if no positive bias is applied, the re-oxidation takes a longer period of time, and in the case of Hb if

residual O_2 was present in high amounts, this would result in the oxy Fe(II)Hb and not in the Met (Fe(III))Hb observed experimentally.

4.3.3 Stability Study

In order to check the functional stability of the immobilised Hb on the TiO_2 film during reduction and re-oxidation, the reduction/oxidation cycle was repeated up to 8 times. Following figure 4.5, which shows the absorption spectra of DeoxyHb after each cycle, it is noted that the spectral changes were reversible over many cycles of positive and negative applied potentials, that only a relatively small % (~ 20) of Hb is desorbed in total after 8 cycles, and that the protein doesn't get denatured.

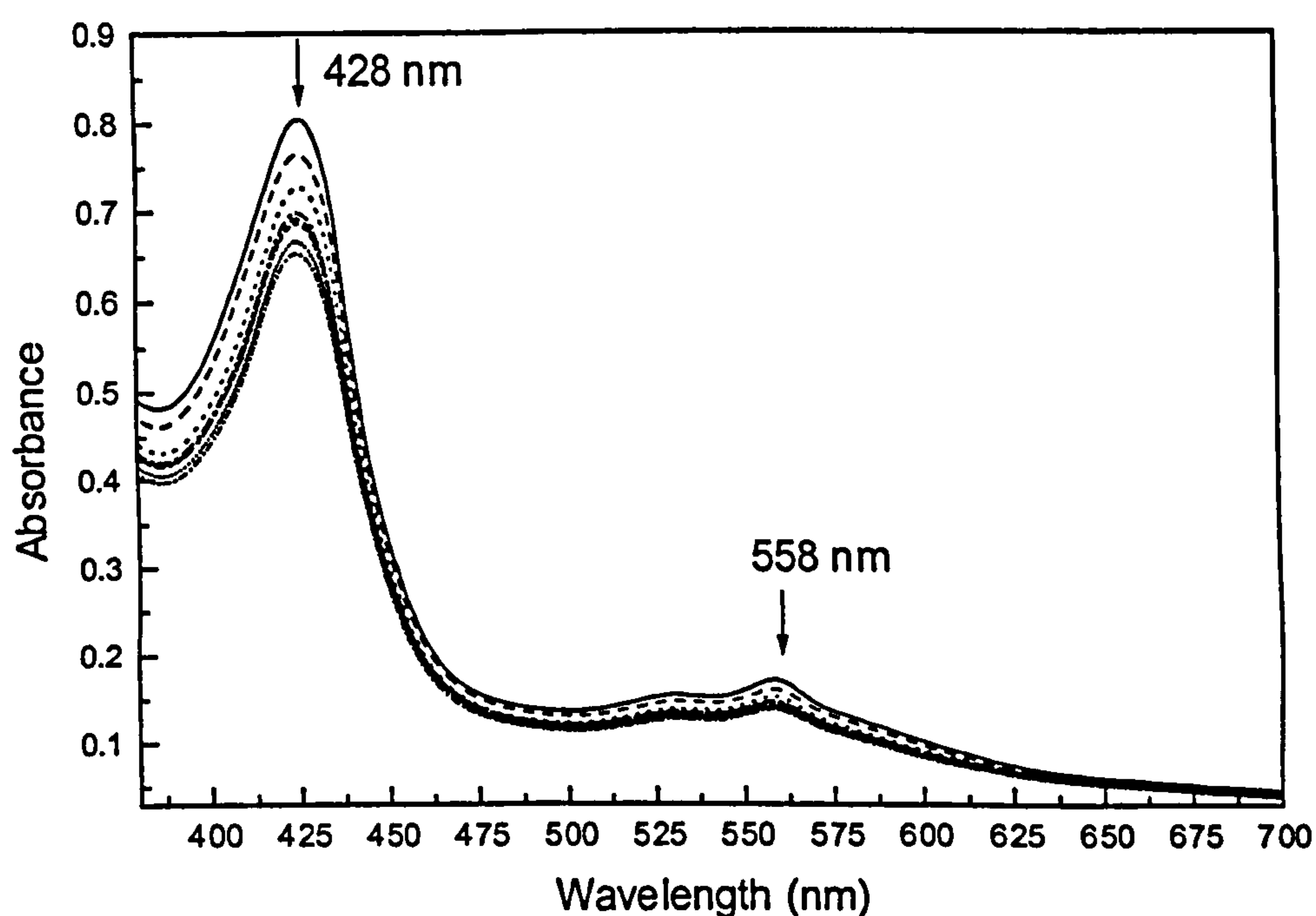


Figure 4.5: UV-vis spectral change of deoxy Fe(II)Hb after 8 cycles of electrochemical reduction and re-oxidation.

During each cycle the immobilised Hb was held at the negative potential (-0.7 V) for 2 minutes. It was noticed that if the time the immobilised Hb was held at the negative bias was increased to 10-15 minutes the amount of protein desorbed after 8 cycles was also increased (25-35 %).

4.3.4 Cyclic Voltammetry on TiO₂ films

Cyclic voltammetry was used as an alternative way of examining the electrochemical behaviour of immobilised Cyt-c and Hb on the nanocrystalline TiO₂ films. CV is a useful technique to examine how much conductivity there is between the TiO₂ electrode and the immobilised protein.

Cyclic voltammograms of Cyt-c and Hb immobilised on TiO₂ electrodes show very similar behaviour. Figures 4.6a and 4.6b show the CVs of Cyt-c/TiO₂ and Hb/TiO₂ films respectively in comparison to blank TiO₂ films. In both cases, the blank TiO₂ film shows little current scanning negatively until ca. -0.3 V when a steady increase in the current occurs up until the end of the scan. On the reverse scan the current is peak shaped, falling back to 0 around -0.3 V. The current at the blank film results from the filling and unfilling of electrons in the trap and conduction band sites, as previously described for such films by (Rothenberger et al., 1992, Boschloo et al., 2000). Cyclic voltammograms of protein immobilised on TiO₂ films show cathodic reduction peaks for Cyt-c/TiO₂ and Hb/TiO₂ films at -0.55 and -0.58 V respectively, but neither of them show reverse anodic oxidation peaks.

The solution phase redox potentials for Cyt-c and Hb are +32 mV and -72 mV respectively vs the Ag/AgCl electrode. The shift of the reduction potentials for the immobilised proteins relative to the solution potentials and the lack of reverse oxidation peaks is attributed to the low electrical conductivity of the TiO₂ for potentials <-0.3 V, as I discuss in detail later in this chapter.

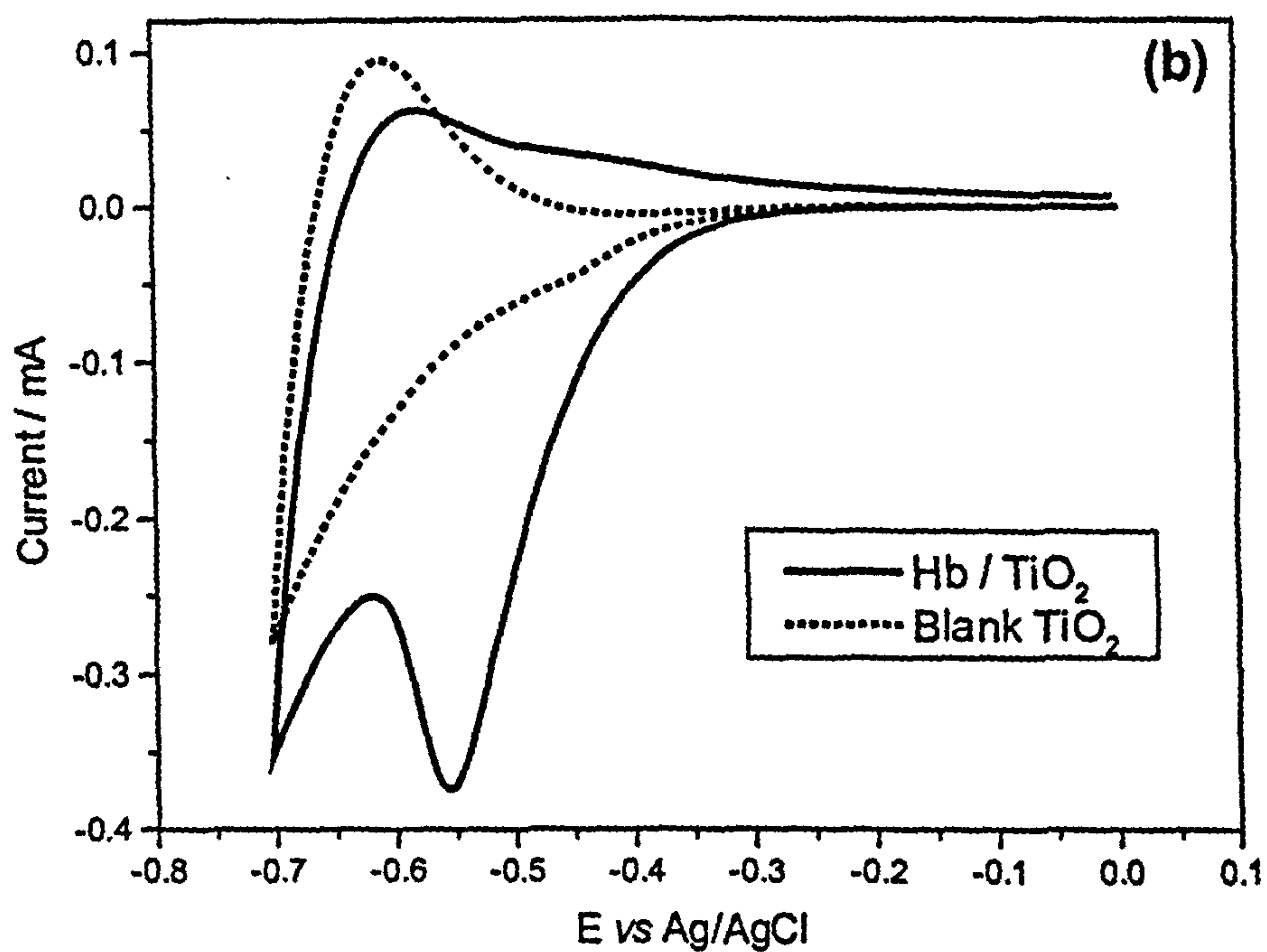
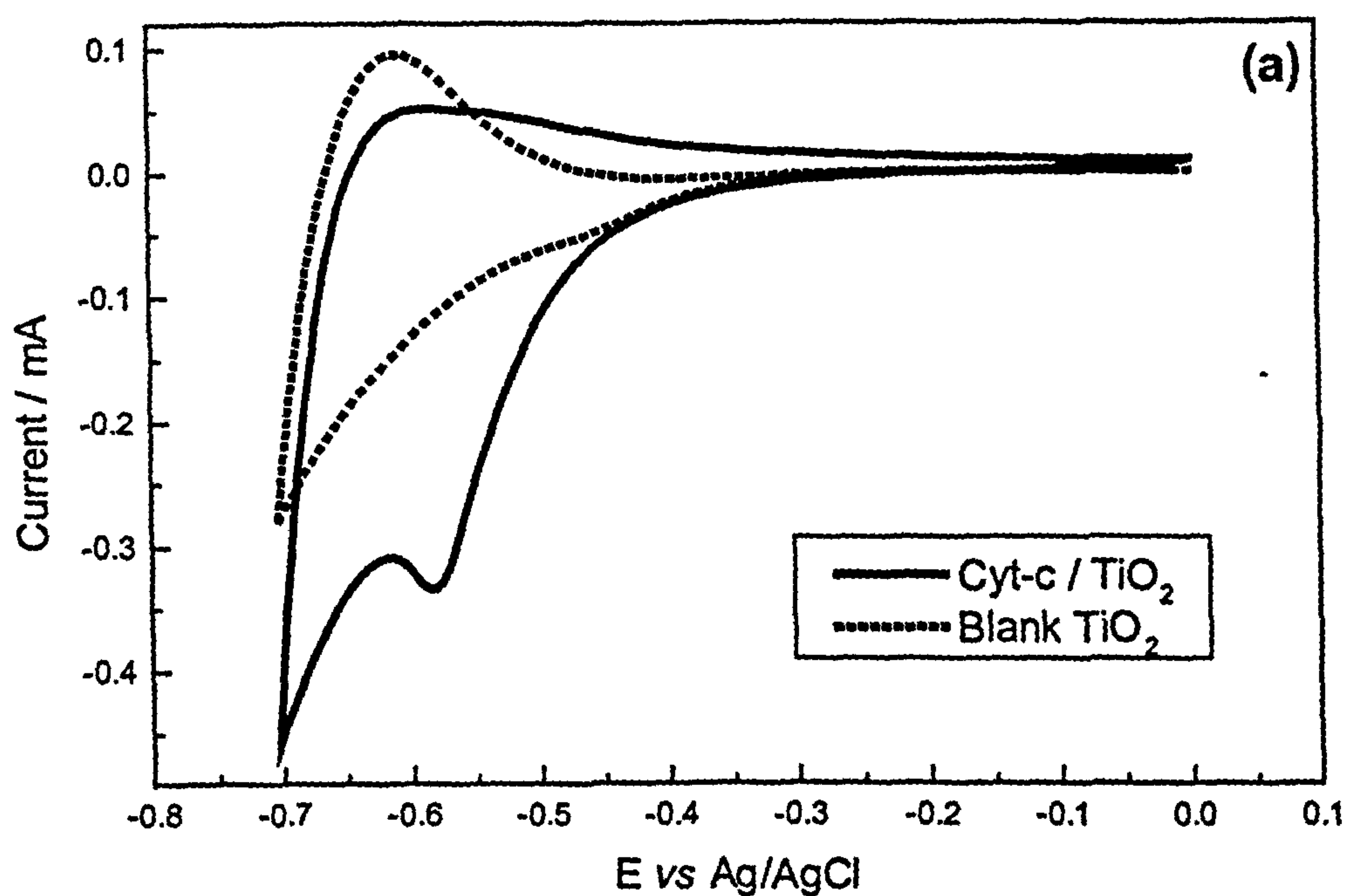


Figure 4.6 : Cyclic voltammograms at 0.1 V s^{-1} in a pH 7, 10 mM NaH_2PO_4 buffer for (a) a Cyt-c/TiO₂ film; and (b) a Hb/TiO₂ film, in comparison to a blank TiO₂ film.

The negative shift between the observed reduction peaks from the CVs of the two proteins immobilised on TiO_2 films and the redox potentials observed in solution is attributed to the low conductivity of TiO_2 films (Rothenberger et al., 1992) at moderate potentials (will be discussed in detail later) or due to a shift in the redox potential of the proteins induced by immobilisation. In order to investigate this, a number of control experiments were conducted.

Using a simple solution phase redox system ($\text{Fe}(\text{CN})_6$) and a blank TiO_2 working electrode it was attempted to point out that although ($\text{Fe}(\text{CN})_6$) is not immobilised on the TiO_2 film, still the same negative shift in its reduction peak should occur and therefore voltammetry is interfered by the conductive properties of the TiO_2 film. Figure 4.7 confirms this expectation as it shows a very similar voltammetric behaviour for ($\text{Fe}(\text{CN})_6$) in solution to that observed for the Cyt-c/ TiO_2 film due to the low conductivity of the TiO_2 at moderate potentials.

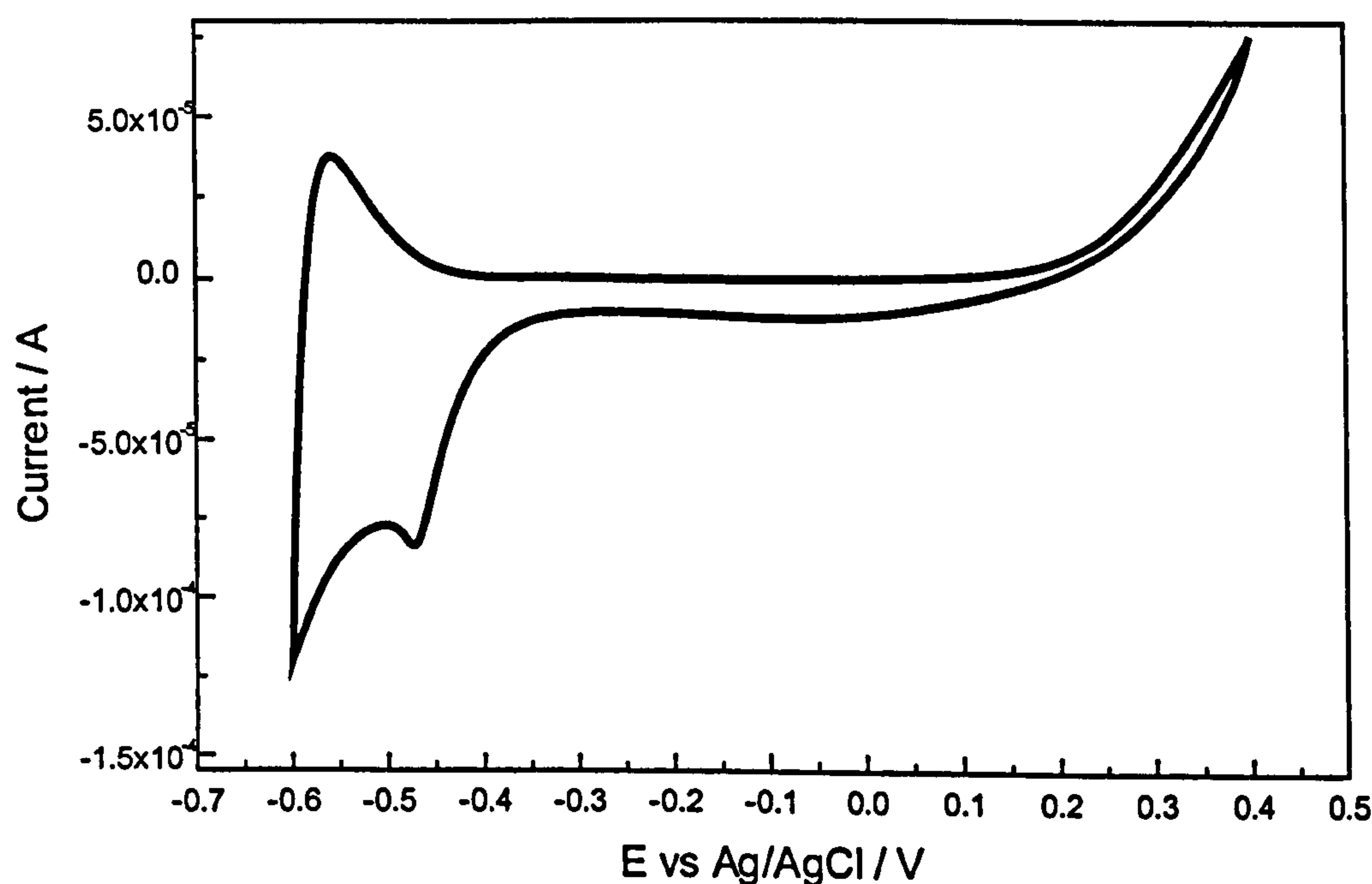


Figure 4.7: Cyclic voltammogram for a blank TiO_2 film in $\text{Fe}(\text{CN})_6$ solution.

Alternatively slower scan rates were applied to the Cyt-c/TiO₂ electrode in order to obtain a reversible peak shape CV. Figure 4.8 shows that even at slow scan rates no simple reversible behaviour for the Cyt-c/TiO₂ film was observed, consistent with the currents being limited by the low TiO₂ conductivity at moderate potentials.

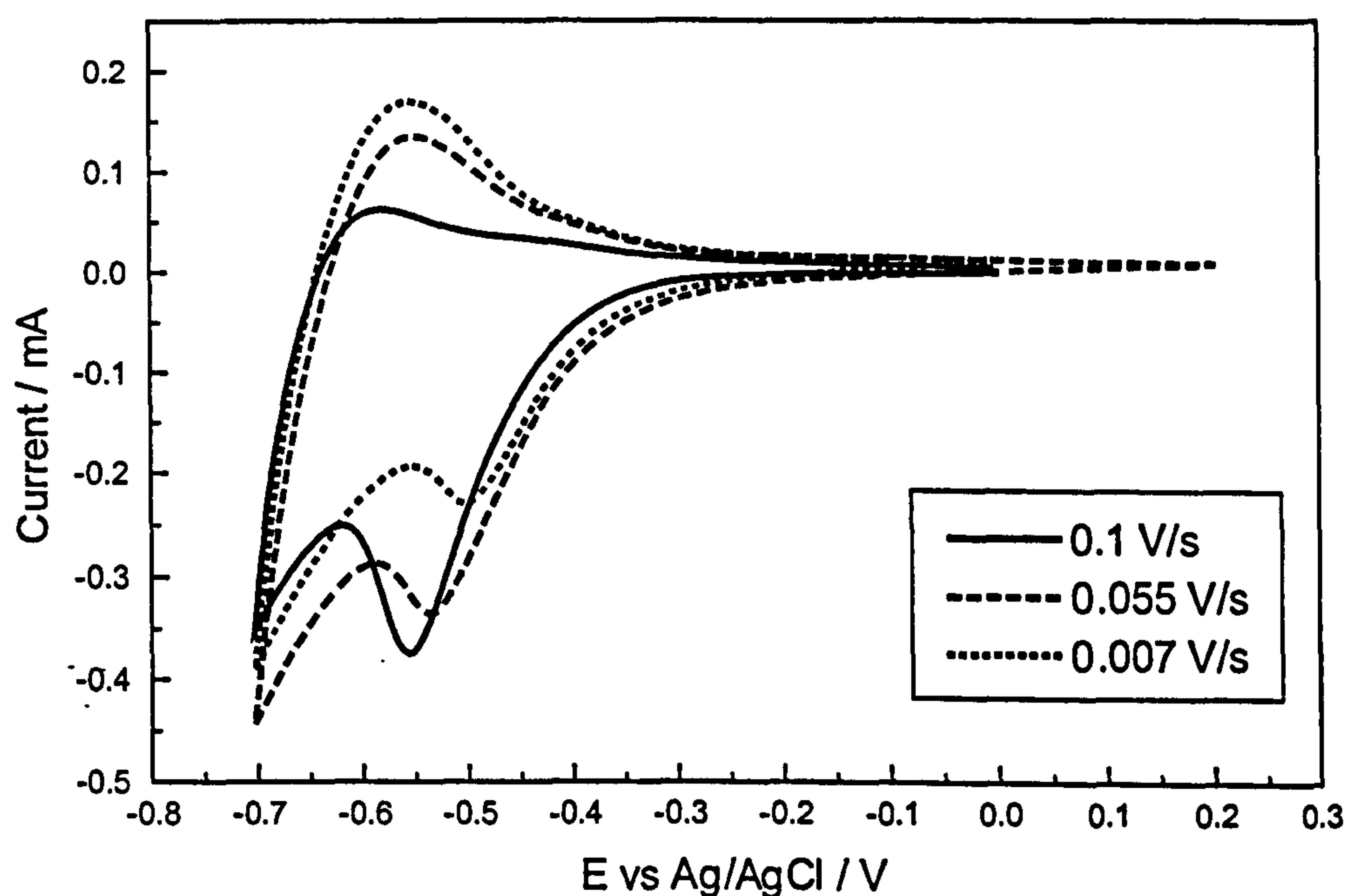


Figure 4.8: Cyclic voltammograms for a Hb/TiO₂ film in a pH 7, 10 mM NaH₂PO₄ buffer at different scan rates (V s⁻¹).

4.3.5 Effect of pH

It is known that the pH of the electrolyte solution affects the conductivity of the TiO₂ films. Indeed, the change in pH, by simply altering the HPO₄²⁻/H₂PO₄⁻ ratio but keeping the ionic strength constant, has a significant effect on the potential of the reduction peak for the immobilised Cyt-c and Hb, as can be seen in figures 4.9a and 4.9b respectively. The CVs of these figures show that by lowering the pH by 1.7 units (pH 7 to 5.3), the potential of the reduction peak of Cyt-c and Hb changes by 110 mV (ie 64.5 mV/pH unit) and by 108 mV (ie 64 mV/pH unit) respectively due to the change in the conductivity of the TiO₂ films. In both cases the same shift per pH unit is observed. These results agree with the fact that a blank nanocrystalline TiO₂ film in an aqueous electrolyte shows an expected Nernstian shift of ~60 mV on increasing the pH of the electrolyte by a single unit (Boschloo et al., 1999a, 2000). This supports the assignment of the shift of the reduction peak of the immobilised proteins due to the conductivity of the TiO₂ film.

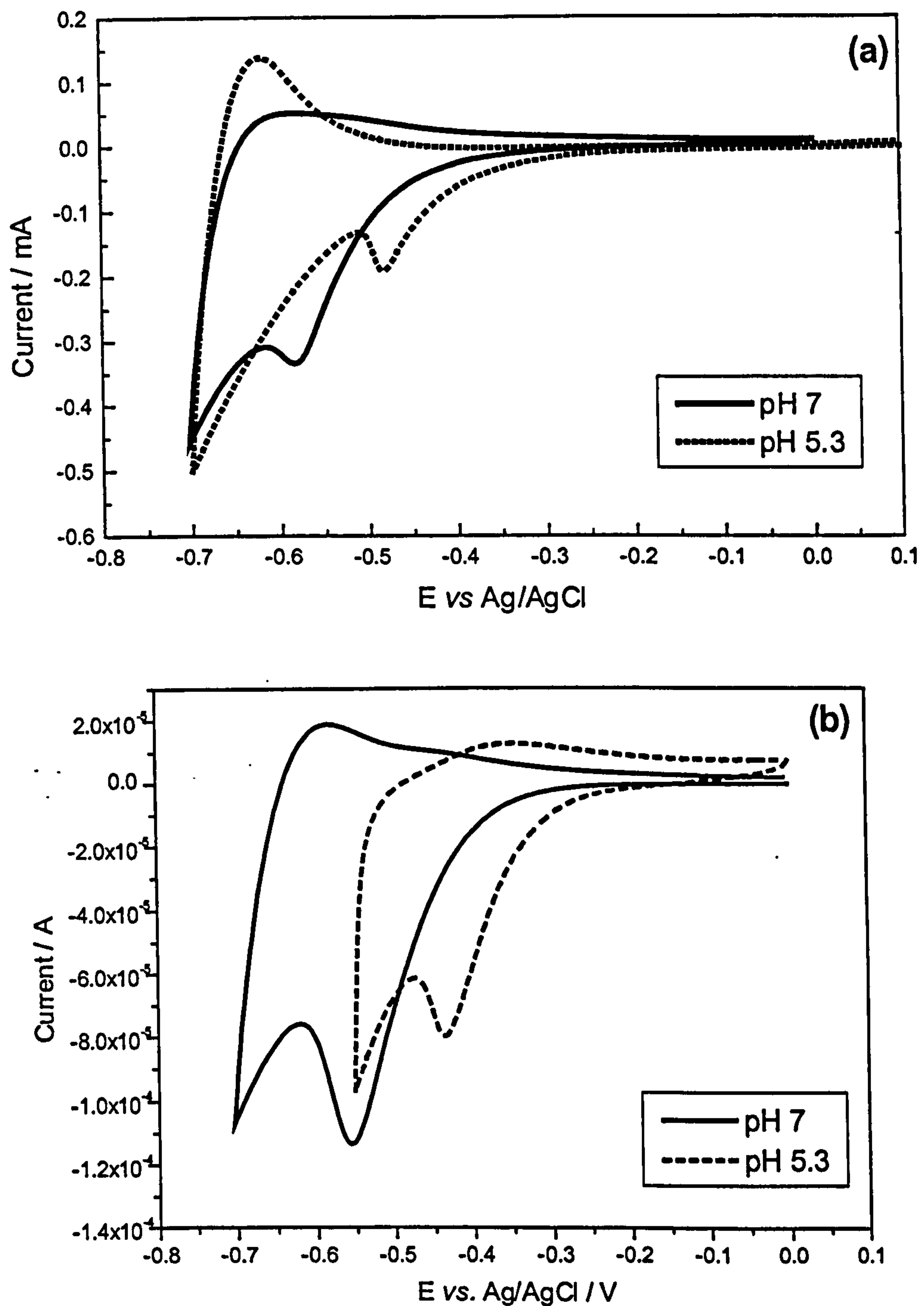


Figure 4.9: Cyclic voltammograms at 0.1 V s^{-1} of (a) a Cyt-c/TiO₂ film in a pH 7 and a pH 5.3, 10 mM NaH₂PO₄ buffer solution; and (b) a Hb/TiO₂ film in a pH 7 and a pH 5.3, 10 mM NaH₂PO₄ buffer solution.

4.3.6 Electrochemical activity of Cyt-c on a ZnO film

Having immobilised Cyt-c successfully on nanocrystalline ZnO films (see Chapter 3), an electrochemical study of the electrochemical properties of the Cyt-c/ZnO film was undertaken and compared to the TiO₂ films. The ZnO film, like the TiO₂ film, is a wide band gap (3.2 eV) semiconductor (Hagfeldt et al., 1995) and therefore optically transparent. Figure 4.10 shows the UV-Vis spectra of the immobilised Cyt-c on the ZnO film after 15 minutes at the given negative potential of (0 to -0.6 V). A clear shift in Soret band from 410 nm to 416 nm and an increase in the sharp α and β bands at 550 and 520 nm are in good accordance with the solution spectrum of the reduced Cyt-c.

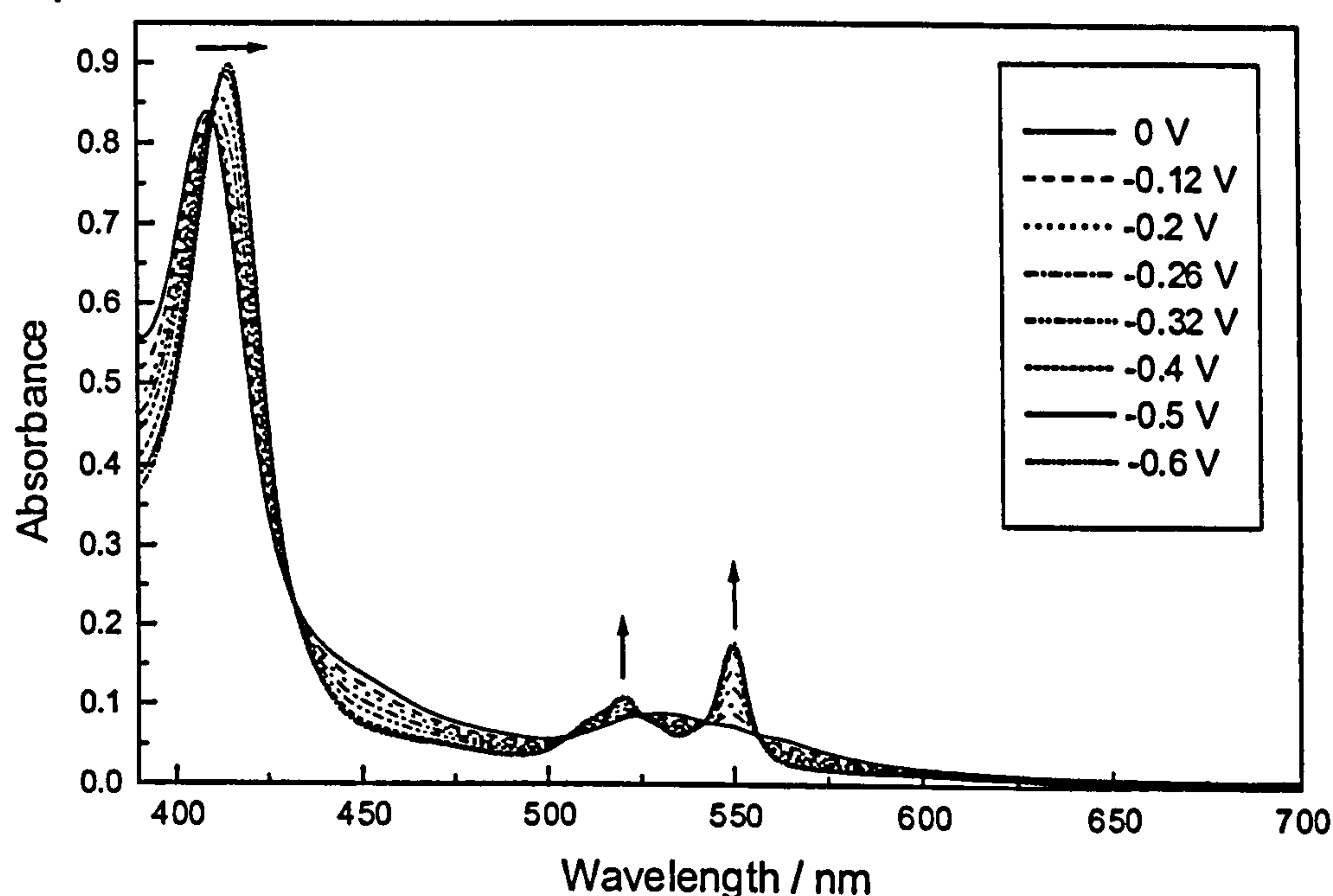


Figure 4.10: UV-Vis spectral change for electrochemical reduction of the oxidised Fe(III) Cyt-c/ZnO at increasing negative potentials (0 to -0.6 V).

Figure 4.11 shows the % of Cyt-c reduced on a ZnO and a TiO₂ film respectively as a function of the applied potential. According to this figure there is a potential dependence of the extent to which Cyt-c became reduced on the ZnO and TiO₂ films. As the applied negative potential is increased higher than -0.12 V the immobilised Cyt-c on the ZnO film starts getting reduced. However, negative potentials up to -0.3 V have no effect on the reduction of Cyt-c on the TiO₂ film.

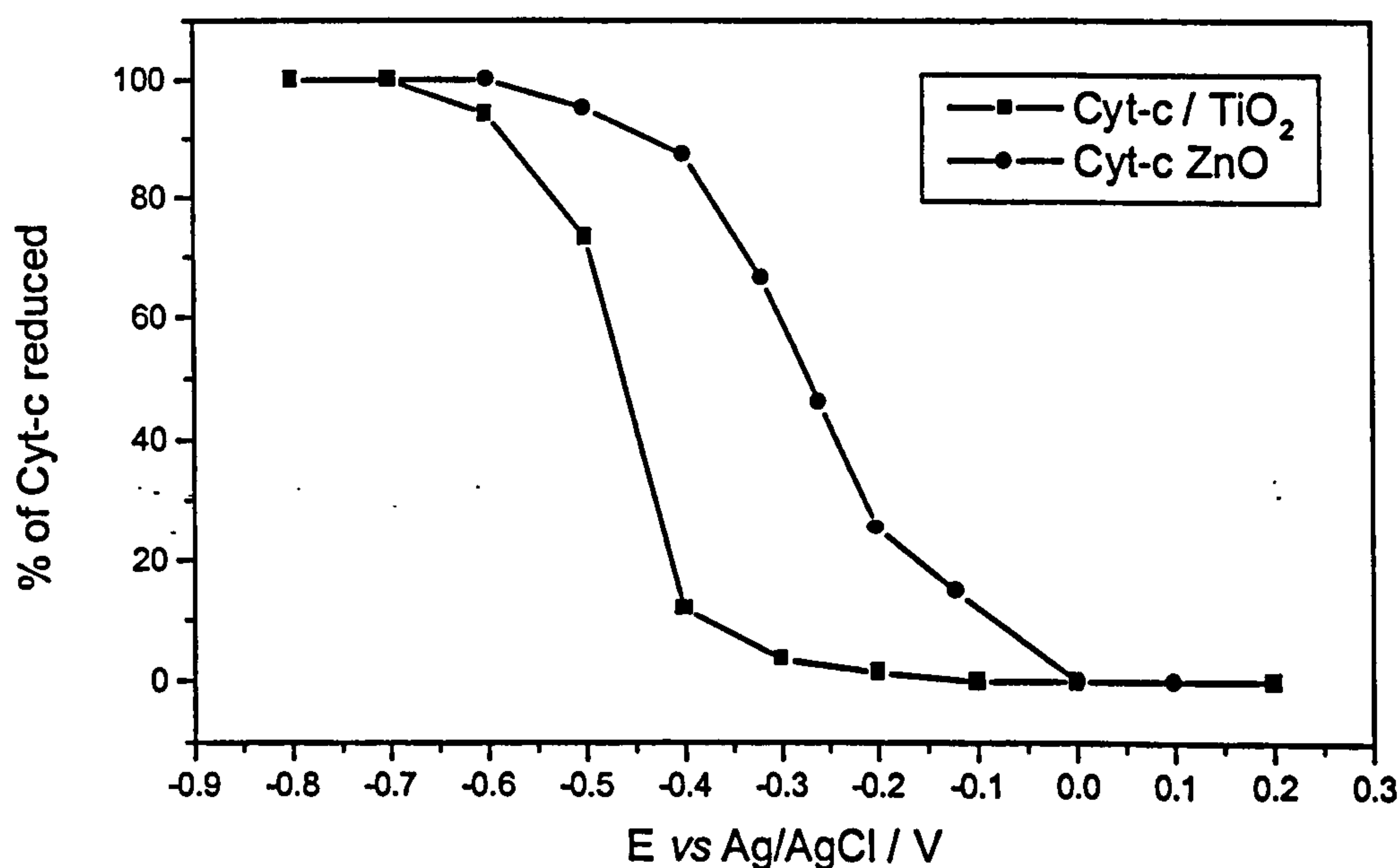


Figure 4.11: % of Cyt-c reduced on a ZnO and a TiO₂ film respectively as a function of the applied potential. Each potential was applied for 15 minutes before the absorbance at 550 nm was recorded.

It is interesting to note that in order to reduce the immobilised Cyt-c on the ZnO film it is necessary to apply a significantly less negative potential than those applied on a Cyt-c/TiO₂ film. The electrochemical reduction of Cyt-c on the ZnO electrode starts at -0.12 V, a potential at which Cyt-c remains at its Fe(III) oxidised state on the TiO₂

electrodes. Also at -0.4 V only 10% of Cyt-c was reduced on the TiO₂ film compared with the 90% protein reduction achieved on the ZnO film due to a high density of intraband defect states. This is attributed to an improved conductivity of the ZnO films compared to the TiO₂ films at moderate applied potentials, consistent with recent chronoamperometric studies of these films (Willis et al., 2001). However, a more negative bias is still applied than those expected from solution phase redox chemistry of Cyt-c.

As for the Cyt-c/TiO₂ films, the immobilised Cyt-c on the ZnO films is re-oxidised by applying positive potentials (0 to +0.6 V) vs Ag/AgCl and by bubbling argon at the same time.

4.3.7 Cyclic voltammetry on ZnO Films

Cyclic voltammetry was used as an alternative way to characterise the nanocrystalline ZnO film. Figure 4.12 shows the cyclic voltammogram of a 4 µm thick colloidal zinc oxide film, in comparison to a TiO₂ film, in pH 7 buffer solution. This is the same cyclic voltammogram observed in the past by Hoyer et al., 1995 and Lemon et al., 1997. The blank ZnO film shows only a minor current response between +0.6 and -0.12 V, while at more negative potentials (> -0.18 V), it becomes strongly charged. The current results from accumulation of electrons in the trap and conduction band sites of the film. Compared to the cyclic voltammogram of a blank TiO₂ film, ZnO films show much better conductivity at moderate potentials and more reversibility due to lack of H⁺ intercalation (Lemon et al., 1997).

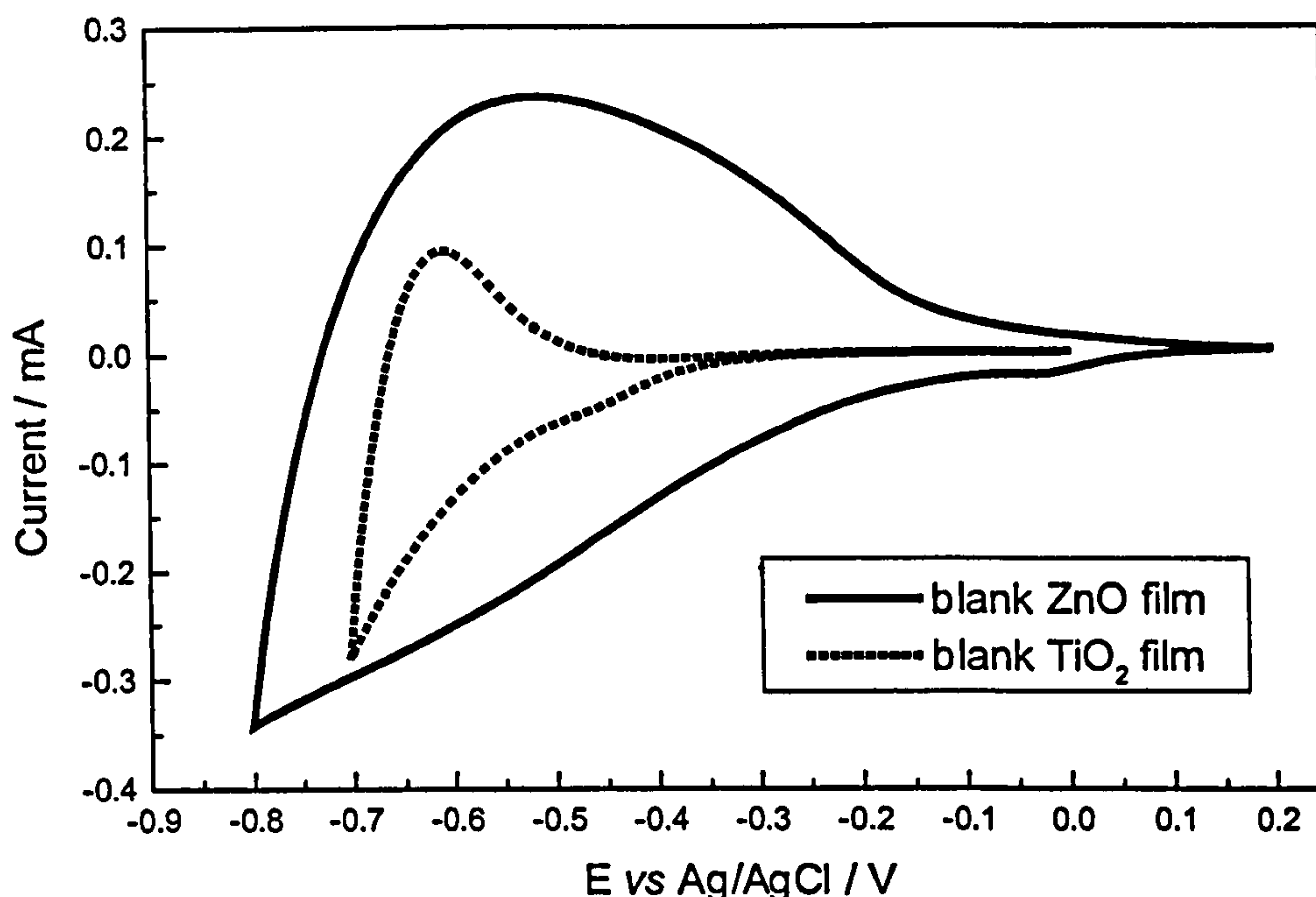


Figure 4.12: Cyclic voltammograms at 0.1 V s^{-1} in a pH 7, 10 mM NaH_2PO_4 buffer for a blank ZnO and a blank TiO_2 film.

4.3.8 Spectroelectrochemical studies of blank TiO_2 films

In order to further examine the conductivity of the TiO_2 films spectroelectrochemical techniques were used. These techniques take advantage of the facts that thin nanostructured TiO_2 films may be deposited on conducting glass supports to yield transparent electrodes and that electrons present in these electrodes have an optical spectroscopy characteristic of their local environment. Following the procedure of (Rothenberger et al., 1992) spectroelectrochemical data were collected using 10 mM NaH_2PO_4 pH 7 as the electrolyte for blank TiO_2 films. Shown in figure 4.13a are the potential dependent optical absorption of a nanoporous blank TiO_2 electrode measured

at the indicated potentials in 10 mM phosphate buffer solution of pH 7. At low and moderate negative applied potentials the measured absorption spectra is pretty much the same to the one where no potential is applied. At still more negative potentials, however, a broad absorption maximum is observed at about 750 nm.

Figure 4.13b follows the absorption increase at a single wavelength (750 nm) as a function of the applied bias. Although the increase in absorbance is higher the higher the negative potential applied, the kinetics of these increases are the same (10-15 seconds).

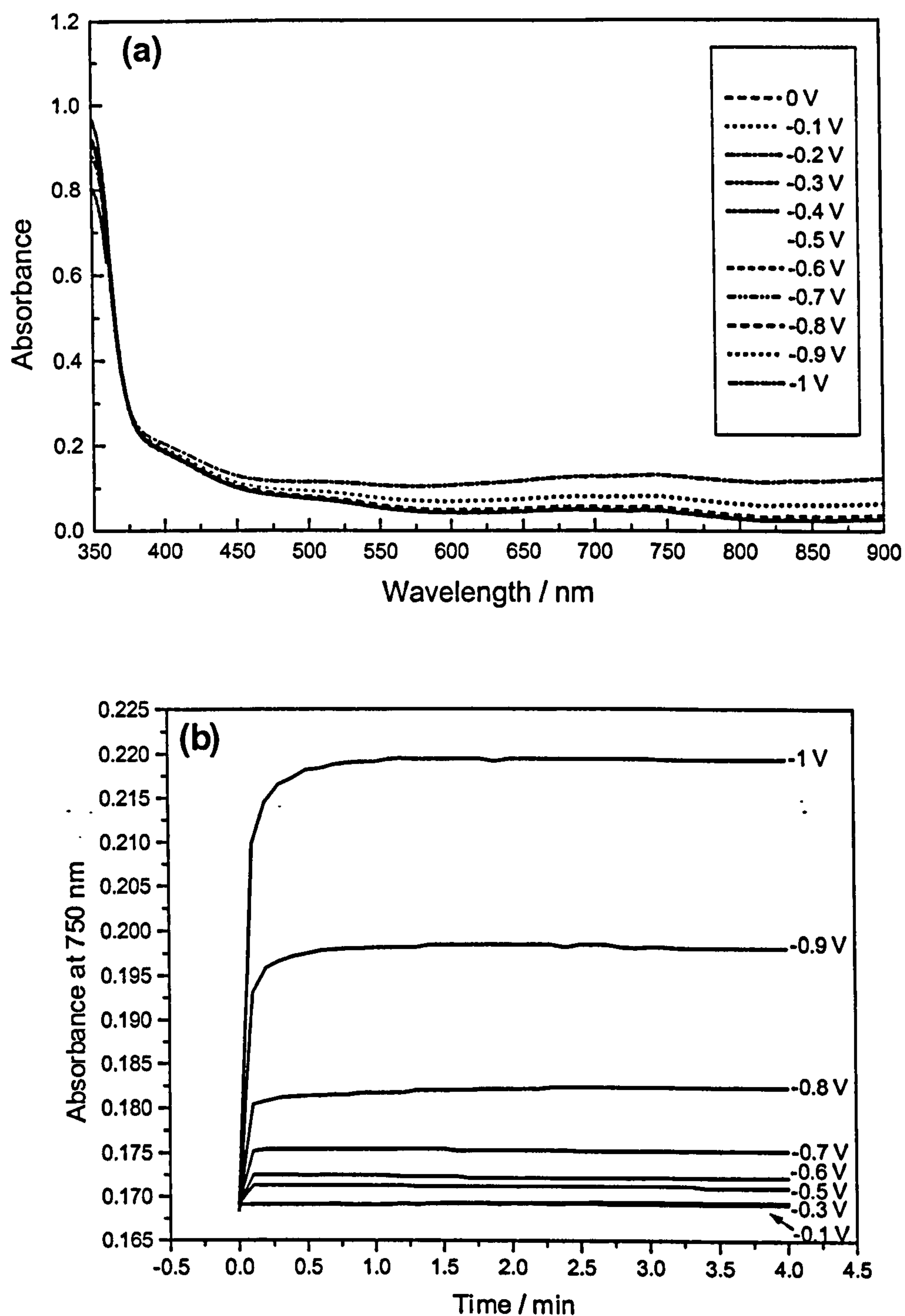


Figure 4.13: Spectroelectrochemical data showing (a) the absorption spectrum of a blank TiO_2 electrode in 10 mM phosphate buffer solution, pH 7, measured at 0 to -1V vs Ag/AgCl reference electrode. (b) the time dependent absorbance changes at 750 nm for a blank TiO_2 electrode measured at measured at -0.1 to -1V vs Ag/AgCl reference electrode.

Figure 4.13b follows the absorption increase at a single wavelength (750 nm) as a function of the applied bias. From figure 4.14 it is obvious that the data exhibit an approximately exponential tail towards the more positive biases. It has been suggested that this tail may be due to the presence of sub-band gap defect states (Rothenberger et al., 1992). This increase in absorbance relates to the electron density per nanoparticle.

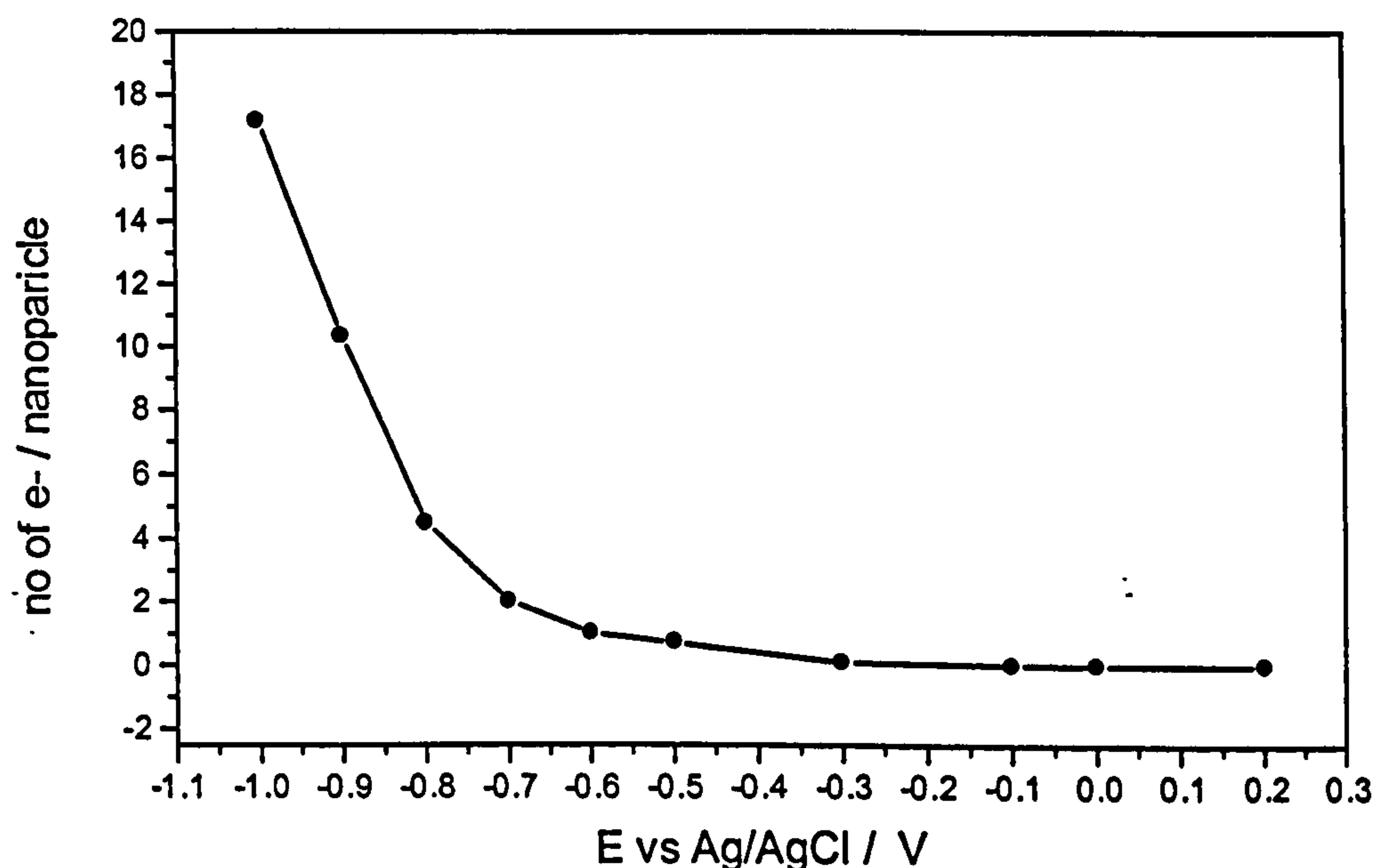


Figure 4.14: Electron density in a blank TiO_2 electrode as a function of bias.

The electron density can be determined from the spectroelectrochemical data. The increase in electron density in the film can be determined by monitoring the increase in absorption at 750 nm as a function of externally applied bias. It has been suggested that the coloration (blue/black) of the TiO_2 film under negative applied bias is due to the filling of the trap/conduction band states. Therefore the change in absorbance at 750 nm

can be related to the electron density n . The change in absorbance A is defined by the Beer-Lambert law:

$$A = \varepsilon cl \quad (1)$$

Where ε is the extinction coefficient of an electron at 800 nm, $1.3 \times 10^{21} \text{ m}^2$ (Rothenberger et al., 1992), c is the concentration and l the path length. Equation 1 can be used to derive the electron density n per nanoparticle by substituting c with n/V . This is given by the equation 2.

$$n = \frac{AV}{\varepsilon l} \quad (2)$$

Where V is the volume of the TiO_2 film, $8 \times 10^{-10} \text{ m}^3$ and l is the thickness of the TiO_2 film, $8 \times 10^{-6} \text{ m}$ and n is the electron density. It is estimated that there are 2.25×10^{14} nanoparticles in a film volume of $8 \times 10^{-10} \text{ m}^3$ assuming 15 nm sized particles in an 8 μm thick film.

According to figure 4.14, at $-0.3 \pm 0.05 \text{ mV}$ the electrons start filling the trap/conduction band states of the blank TiO_2 film at a rate of 0.1 e^- per nanoparticle. This rate increases as the negative applied potential is increased reaching a value of 17.5 e^- per nanoparticle at -1 V . Therefore, these results confirm the limited conductivity through the TiO_2 films at low potentials.

The same spectroelectrochemical experiments were performed on Hb/ TiO_2 and Cyt-c/ TiO_2 films. According to figure 4.15 it is obvious that the reduction rate (discussed previously) of both adsorbed heme proteins is consistent with the conduction these films show.

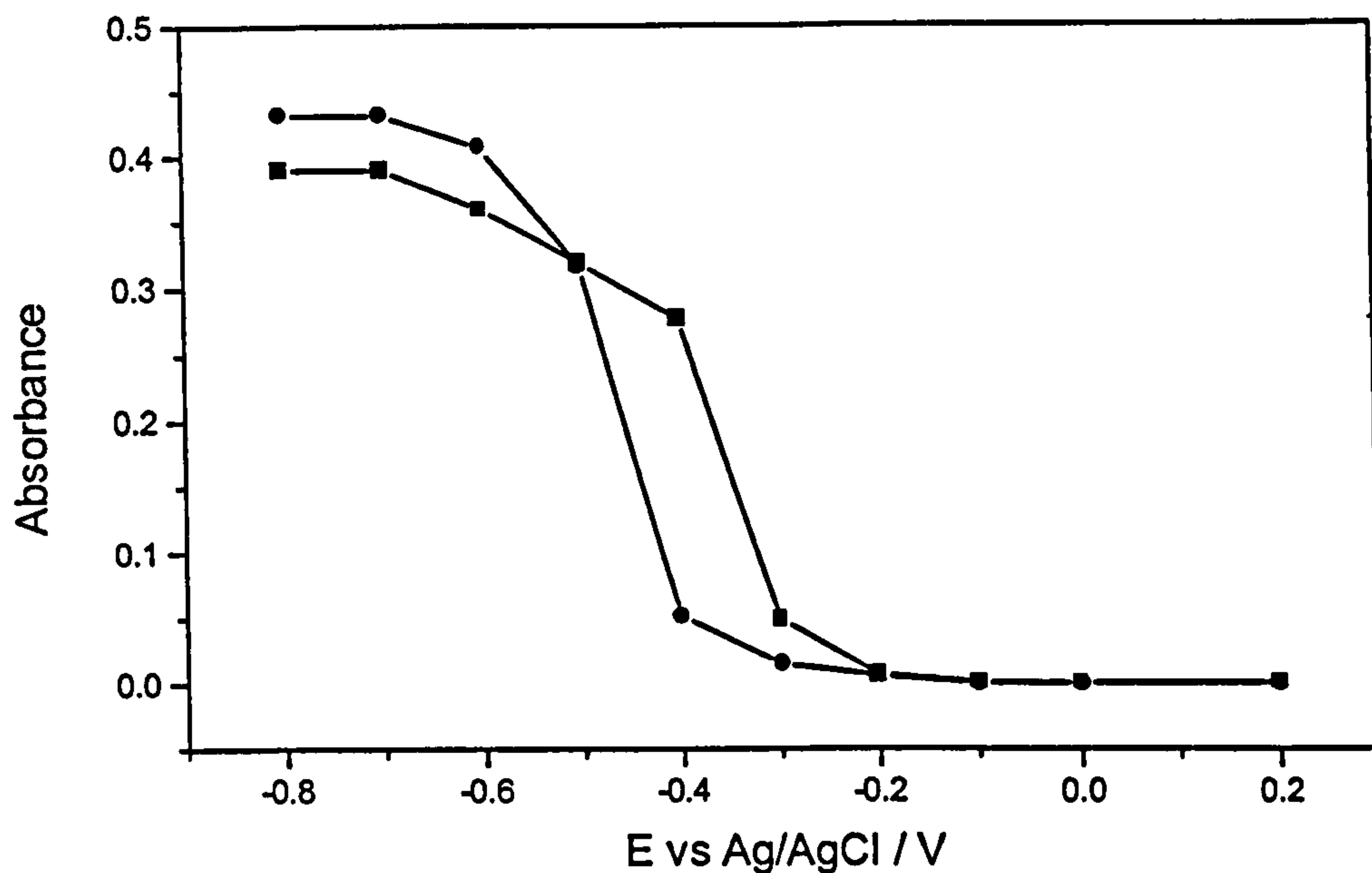


Figure 4.15: Optical absorbance at 550 nm for Cyt-c/TiO₂ (●) and 428 nm for Hb/TiO₂ (■) measured as a function of the applied potential. Each potential step was applied for 15 minutes before the absorbance was recorded.

At low biases (0 to -0.3 V) there is no conduction occurring and therefore the adsorbed proteins remain in their Fe⁺³ oxidation state. At biases higher than the -0.3 V both proteins start getting reduced (Fe⁺²) and the amount of protein reduction increased as the negative applied bias was increased reaching saturation (all the adsorbed protein is reduced) at -0.7 V. Therefore it is concluded that the reduction kinetics of the adsorbed proteins pretty much match the growth of Ti³⁺ absorbance measured with no protein molecules adsorbed on the films.

4.4 General Discussion

Electrochemical activity: Bioelectrochemical studies of redox proteins on electrodes typically require the use of small molecules to promote or mediate electron transfer between the protein redox site and the electrode surface. However, it was found relatively easy to achieve the electrochemical reduction and oxidation of Cyt-c and Hb immobilised on nanoporous TiO₂ electrodes without the addition of any redox promoters or mediators (Topoglidis et al., 1998 and 2000). Application of a negative potential (-0.7 V vs Ag/AgCl) results in reduction of *Fe(III)* Cyt-c or Hb to *Fe(II)*, subsequently the application of a positive potential (0.4 V) results in reoxidation of the Cyt-c or Hb to *Fe(III)*. The absorption spectra of the adsorbed *Fe(II)* and *Fe(III)* Cyt-c or Hb are indistinguishable from their solution spectra, indicating that proteins retain their native conformational state throughout the redox cycle.

It is interesting to note that all the immobilised Cyt-c or Hb are electrochemically reducible whether the thickness of the TiO₂ films are 4 or 8 µm. Investigations into more densely packed layer-by-layer films of proteins and polyions show incomplete protein reduction in ultra thin films (5-10 nm), the authors finding that in the assemblies of multilayers of protein and polyions, only approximately 1.5 layers of protein are electroactive (Lvov et al., 1998). The same authors subsequently reported the assembly of thicker but more porous assemblies of layers of manganese dioxide nanoparticles with alternate layers of myoglobin and polyions (Lvov et al., 2000). It was found that with nanostructured films they could make up to 10 layers of protein and still retain significant electroactivity. It is possible that the more open structure of nanostructured films allows better electron transfer in the direction perpendicular to the electrode surface because protein molecules in less ordered arrays are in better contact with each

other to exchange electrons than those in alternately stacked layers. However, the TiO₂ films described herein are 20 times thicker than the polyelectrolyte films described previously (Ladam et al., 2001) and the amount of protein adsorbed is 100 times more. In view of this, it is remarkable that on the TiO₂ films all of the Cyt-c or Hb can be reduced and this reduction is attributed to electrical conduction through the nanocrystalline TiO₂ film (Topoglidis et al., 2001).

The reduction / oxidation kinetics are relatively slow (5-90 sec for reduction and 8 to 20 minutes for oxidation, depending upon the applied bias), most probably rate limited by the conductivity of the TiO₂ film below its conduction band edge. Such electrodes are therefore not suitable for cyclic voltammetry studies of electron transfer kinetics but would be quite adequate for a range of bioanalytical/biocatalytic applications.

Hb is a tetramer binding four heme groups. These hemes are more deeply buried in the protein matrix than for Cyt-c, and electron transfer between electrodes and *Hb* is therefore expected to be more inhibited than for Cyt-c, as has been observed in previous bioelectrochemical studies of *Hb* at a range of different electrode surfaces (Cooper et al., 1993, Yang et al., 1999, Katterle et al., 1997). The electrochemical reduction/oxidation of the adsorbed Hb on the TiO₂ films is therefore particularly noteworthy. Electrochemical oxidation/reduction studies of Hb have generally required the addition of mediators/promoters to achieve efficient electrochemistry (Kawahara et al., 1998).

The reduction of both Hb and Cyt-c immobilised on the TiO₂ films required significantly more negative applied potentials than those expected from solution phase redox chemistry of these proteins. The fact that sometimes the commercial proteins are significantly impure cannot explain the high negative potential required for their

electrochemical reduction as similar reduction potentials were observed for both immobilised Cyt-*c* and Hb whether commercial or purified. Under a low negative bias up to -0.3 V, both the immobilised Cyt-*c* and Hb remain in the oxidised state (there is no absorption increase of the reduction bands) although their redox potentials in solution are +38 mV and -72 mV vs Ag/AgCl respectively. As the applied negative bias is increased higher than 0.3 V the protein is gradually reduced. The reduction potential for the immobilised Cyt-*c* or Hb on the TiO₂ films is (-0.7 V) due to the low conductivity of the TiO₂ electrode below its conduction band edge and at more positive potentials. Also it can be tentatively attributed to the influence of the electrostatic charges at the TiO₂ / solution interface. At the solution pH employed (pH 7), the surface of the TiO₂ is expected to be negatively charged, consistent with a negative shift of the protein reduction potential. Further work to address this issue is currently in progress.

The suggestion that the electron transfer between the TiO₂ film and the immobilised proteins by applying a high negative potential (-0.7 V) is not direct due to reduction of hydrogen or oxygen at the TiO₂ is not true. In the course of the charging behaviour seen for blank TiO₂ films, in the potentials investigated, very little current flows through the solution as is seen from the ratio of the integrated anodic and cathodic currents (integrates minimal Faradaic current) (Boschloo et al., 1999). This is attributed to the fact that the buffer used is thoroughly purged of oxygen and it is not until significantly more negative potentials than those applied that reduction of protons occurs at TiO₂ (Serpone et al., 2000). This is strong evidence that the reduction of protein that is observed on the TiO₂ electrodes is the result of direct electron transfer from the TiO₂ electrode to the protein and not through the generation of either hydrogen peroxide or hydrogen at the electrode or because mediators or promoters were added.

The reduction of the adsorbed proteins on the TiO_2 films require a negative potential higher than -0.3 V due to the low conductivity of the films. When the applied biases are in the range of -0.3 to -0.6 V, the adsorbed protein starts getting reduced and the reduction kinetics are faster the higher the applied bias. Reduction should still in theory reach completion for potentials $< E_m$ of the adsorbed protein. However it is noticed that this completion is not reached. The reasons could be that different proteins behaving differently due to either different conformation they take after they are adsorbed, how exposed and in direct contact with the electrode the heme is or due to variations in potential felt by the TiO_2 electrode. The latter means that how fast the electrons travel in the electrode and if they get trapped or not depends on the applied bias and therefore they are able to reduce some of the adsorbed protein molecules but not all of the them if the applied bias is lower than -0.7 V.

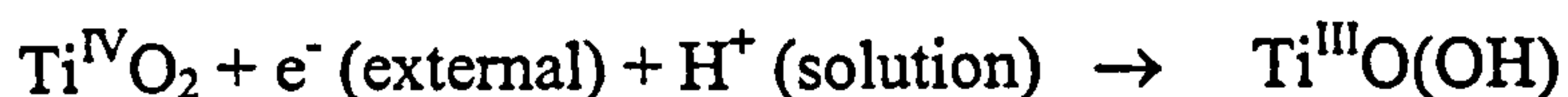
Stability: The stability of the immobilised Hb during the redox cycles is quite good. Even after many redox cycles, the immobilised Hb is reasonably stable, in its native state, the Hb unbinding is very low, it retains its redox activity and shows functional stability. These characteristics of the immobilised Hb are particularly encouraging because they are key issues for biotechnological applications, such as enzyme based biosensors.

Cyclic voltammetry: Voltammograms of the Cyt-c/ TiO_2 and Hb/ TiO_2 films show irreversible electron transfer from the TiO_2 film to both proteins above a threshold of ~ -0.3 V (Topoglidis et al., 2001). This is a substantially more negative value for protein

reduction than would be expected on the basis of their solution redox potentials. The assignment of the peaks showed in figure 4.6 is confirmed also by the spectroelectrochemistry that was carried out on these films in the previous section of this chapter. The insulating region which results (at potentials positive of -0.3 V) because of the large band gap of the TiO₂ films also explains why scanning in the positive direction results in no peak due to protein re-oxidation. At potentials at which the protein would be expected to be oxidised it is well insulated by the TiO₂ film because the fermi level of the film lies well within its bandgap and its conductivity is therefore very low.

Finally, the change in pH of the electrolyte solution has a ~ -64 mV per pH unit shift on the potential of the reduction peak of the immobilised proteins and therefore the well-known dependence of the conduction band edge of TiO₂ films on pH (shifts of approximately -60 mV per pH unit (Boschloo et al., 2000) was confirmed.

Conductivity: The potential induced optical changes in nanostructured TiO₂ films are mainly due to accumulation of electrons in conduction band states. Using this band filling model, it is possible to explain the observed spectra. At low negative applied potentials up to -0.3 v the optical absorption spectra of a nanostructured TiO₂ film is the same as of an unbiased one. At more negative potentials a pronounced absorption maximum develops at 750 nm. This absorbance increase at 750 nm is assigned to intra- and interband transitions by electrons accumulated in conduction band states. When high negative potentials are applied to the TiO₂ electrodes electron accumulation and H⁺ intercalation is accompanied by band filling according to the following equation:



That is, electrons occupy conduction band states with the accumulated charge being compensated by adsorption of a proton from the aqueous electrolyte (Boschloo et al., 1999a).

Previous work (Boschloo et al., 2000) in addition to our own studies has shown that in aqueous buffer at pH 7 trap filling effects and electron accumulation in TiO_2 films occurs above -0.3 V. This is due to the large band gap (3.2 eV) for this material and explains why both of the proteins studied are reduced at more negative potentials than might be expected. The insulating region which results (at potentials positive of -0.3 V) from this large band gap also explains why scanning in the positive direction results in no peak due to protein re-oxidation. At potentials at which the protein would be expected to be oxidised it is well insulated by TiO_2 .

Comparison to ZnO films: Voltammograms of the Cyt-c/ ZnO films show electron transfer from the film to the protein above a threshold of -0.15 V. However, ZnO films show a smaller insulating region than the TiO_2 films and although a more negative value is necessary for protein reduction than would be expected according to protein solution phase redox potential, still they show better conductivity than the TiO_2 films and more reversibility due to lack of H^+ intercalation. Also according to spectroelectrochemical data collected for the Cyt-c/ ZnO film spectroscopic changes were observed characteristic of the reduction of the iron heme moiety of the immobilised Cyt-c. Therefore, the ZnO films not only they show the properties of the TiO_2 films but also because of their better conductivity can offer a better surface for studying the electron

transfer kinetics between the electrode and the protein and also develop faster and more reliable electrochemical biosensors.

4.5 Conclusions

In this chapter the electrochemical properties of the nanoporous TiO₂ films were characterised. It is clearly demonstrated that the proteins studied here retain their electrochemical activity, characterised by both UV/Vis spectroelectrochemistry and cyclic voltammetry. However, the TiO₂ films have an insulating region at low negative potentials and this is attributed to their limited conductivity at such potentials. The significance of these studies is that TiO₂ films can be used further in the design of novel optical or electrochemical biosensors or for studying protein/electrode interactions.

CHAPTER 5

AN INITIAL STUDY OF THE ELECTRON TRANSFER KINETICS BETWEEN TiO_2 ELECTRODES AND THE ADSORBED PROTEINS USING TRANSIENT ABSORPTION SPECTROSCOPY

5.1 Introduction

Electron transfer kinetics between electrodes and adsorbed redox proteins are typically studied by cyclic voltammetry (Hirst et al., 1998). Under optimal conditions and employing fast scan speeds, electron transfer rate constants of up to $\sim 10^4 \text{ s}^{-1}$ have been reported, although the interpretation of such voltammetry data is often complicated by the observation of non-ideal voltammograms. Such non-ideal behavior has been attributed to irreversible adsorption, protein denaturation and presence of diffusion processes (Taniguchi et al., 1997 and Rusling et al., 1998).

However, according to Armstrong et al., 2000a by carrying out cyclic voltammetry over a wide range of scan rates and exploiting the ability to poise or pulse the electrode potential between cycles, data can be obtained that are conventionally analysed in terms of plots of peak potentials against scan rate. A simple reversible electron transfer process gives rise to a 'trumpet'-shaped plot because the oxidation and reduction peaks separate increasingly at high scan rate and the electrochemical kinetics can then be determined. Using this method results have been obtained for some proteins (e.g. ferredoxin) which show that it is possible to get near 'ideal' behaviour (simple and uncoupled electron transfer) (Armstrong et al., 2000b).

Unfortunately, cyclic voltammetry cannot be used to study the electron transfer kinetics between the adsorbed protein (Cyt-c or Hb) and the TiO_2 electrode due to the limited conductivity of the TiO_2 films below their conduction band edge (Boschloo et al., 1999a and Rothenberger et al., 1992). The relatively slow electron mobility within the nanocrystalline TiO_2 electrodes precludes the use of CV to determine protein/electrode electron transfer kinetics.

In contrast to bioelectrochemical studies of protein redox reactions at electrode surfaces, for solubilised redox proteins the method of choice for kinetic studies of biological electron transfer is transient optical spectroscopy. Such studies are facilitated by the high optical densities readily achieved in a 1 cm optical cuvette. The low optical densities arising from mono-layer coverage of an electrode surface, combined with the fact that most electrodes are not optically transparent, has generally precluded optical studies of protein / electrode electron transfer reactions.

However the optical transparency and high surface area of the TiO₂ films employed in this study opens up the possibility of initiating and monitoring such reactions optically. Indeed, transient optical spectroscopy has been widely employed to determine electron transfer kinetics between such electrodes and adsorbed molecular sensitizer dyes. Following pulsed laser excitation of the sensitizer dye, electron transfer rates of $>10^{12} \text{ s}^{-1}$ have been reported for electron injection from dye excited states into the TiO₂ conduction band (Tachibana et al., 1996). Similarly band gap excitation of TiO₂ nanoparticles by UV light (337 nm) has been widely reported to result in both reductive and oxidative interfacial electron transfers associated with photogenerated conduction band electrons and valence band holes respectively (Kamat et al., 1993). Bandgap excitation of colloidal TiO₂ particles in solution has been reported to result in the photoreduction of Cyt-c (Cuendet et al., 1986). TiO₂ catalysed photochemistry is currently attracting interest for the photodegradation of organic pollutants in water. In this Chapter it is demonstrated that such photochemistry may also be employed to achieve the reduction or oxidation of redox proteins adsorbed to the TiO₂ films.

5.2 Experimental Methods

5.2.1 Preparation of samples

The immobilisation of Cyt-c and Hb on the TiO_2 films was done as described in chapters 2 and 3. Before use the protein/ TiO_2 films were washed with phosphate buffer solution and their absorption spectra was recorded in order to check the redox state of the immobilised proteins.

5.2.2 Steady state photoreduction

Steady state photoreduction of Cyt-c and Hb immobilised on the TiO_2 films was carried out as described in chapter 2 (section 2.5). A drop of 10 mM NaH_2PO_4 buffer solution was added on the films surface using a Pasteur pipette and a very thin circular glass slide was placed on top to cover the sensitive surface area of the film. Then the protein/ TiO_2 film was positioned into the sample compartment of the Shimadzu UV1601 spectrophotometer and was illuminated by the filtered (350 nm) output of a 150 W Xenon lamp for a few minutes using a light pipe. The absorption spectrum of the protein/ TiO_2 film was monitored during and after the illumination.

5.2.3 Transient Absorption spectroscopy

Microsecond-millisecond transient absorption spectroscopy was performed as described in chapter 2 (section 2.6). Initially, transient absorption changes were recorded 1 μ s after pulsed laser excitation of the Fe(III) Cyt-*c*/TiO₂ Fe(III) and Hb/TiO₂ films at different probe wavelengths (535-600 nm). Photoreduction kinetics were obtained on the immobilised Fe(III) Cyt-*c* in the presence of 3 mM potassium ferricyanide so that the heme got reoxidised between laser pulses. A microsecond time scale was used. On the other hand photooxidation kinetics were obtained by pre-reducing the immobilised Cyt-*c* to Fe(II) by longed exposure to the laser excitation pulses (10-20 pulses) in the absence of ferricyanide using the same time scale.

5.3 Results and Discussion

Figure 5.1 shows the schematic representation of the photoinduced electron-transfer processes between the nanoporous TiO₂ particles and the heme (H) group of Cyt-*c* (Topoglidis et al., 2000). Absorption of UV light (< 350 nm) by the semiconductor film results in the generation of conduction band electrons and valence band holes. Depending upon the initial redox state of the adsorbed protein, the protein may be reduced by the conduction band electrons or oxidised by the valence band hole. The efficiency of these reactions depends upon kinetic competition between electron/hole transfer to the protein, electron/hole transfer to alternative electron donors/acceptors in solution and charge recombination within the TiO₂ film.

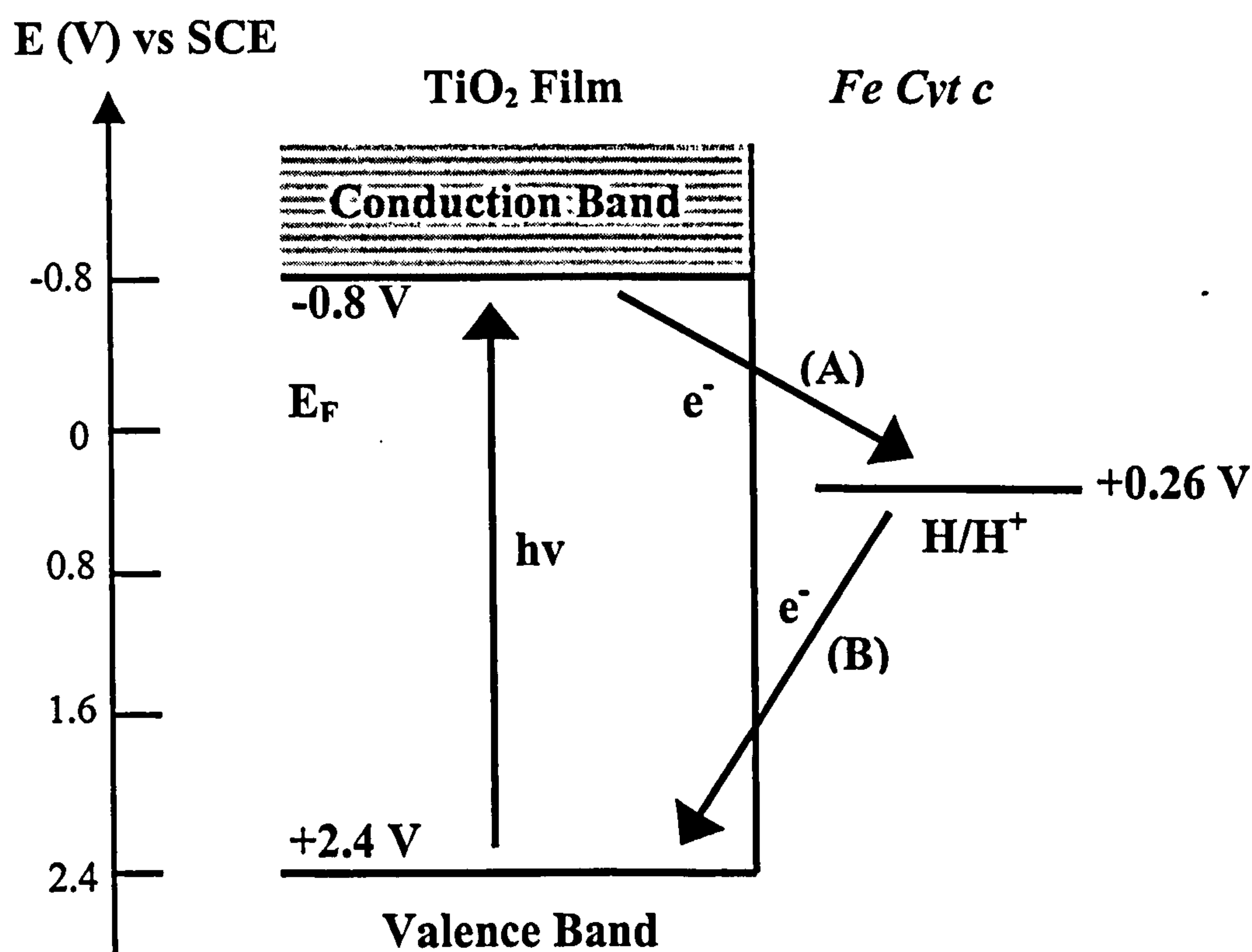


Figure 5.1: Schematic representation of the photoinduced electron-transfer processes between the nanoporous TiO_2 particles and the heme (H) group of $\text{Fe Cyt-}c$; (A): Photoreduction, (B): Photooxidation. H^+ is the oxidised heme. The flat band potential of the semiconductor as a function of the pH value of the phosphate buffer solution (pH 7) is -0.8 V. The band gap for TiO_2 is 3.2 V and therefore the valence band is at +2.4 V.

In practice, the photochemical reduction of $\text{Fe(III) Cyt-}c$ to Fe(II) is more efficient, as illustrated in figure 5.2a. Steady state UV illumination of an $\text{Fe(III) Cyt-}c$ film results in complete reduction of the heme to Fe(II) (Topoglidis et al., 2000). This reduction is reasonably efficient, exhibiting a half time of 30-60 seconds when employing 4.2 mW cm^{-2} of 330 nm light.

Figure 5.2b shows the analogous data obtained for *Hb* / TiO_2 films, showing the photochemical reduction of Fe(III) metHb to Fe(II) deoxyHb, although in this case the half time for reduction of the four heme complex was 60 – 90 s. The greater efficiency of the reductive rather than oxidative photochemistry is attributed to the shorter expected lifetime of the valence band holes, as their high reactivity (valence band edge +2.4 V versus SCE) (Hagfeldt et al., 1995) will allow a wide range of oxidation reactions to solution species in addition to heme oxidation. The photochemical reduction of both proteins was fully reversible in the dark, and did not result in any detectable protein degradation or denaturation.

Figures 5.3a and 5.3b show transient absorption changes observed 1 μs after pulsed laser excitation of Fe(III) Cyt-*c*/ TiO_2 and Fe(III) Hb/ TiO_2 films respectively (Topoglidis et al., 2000).

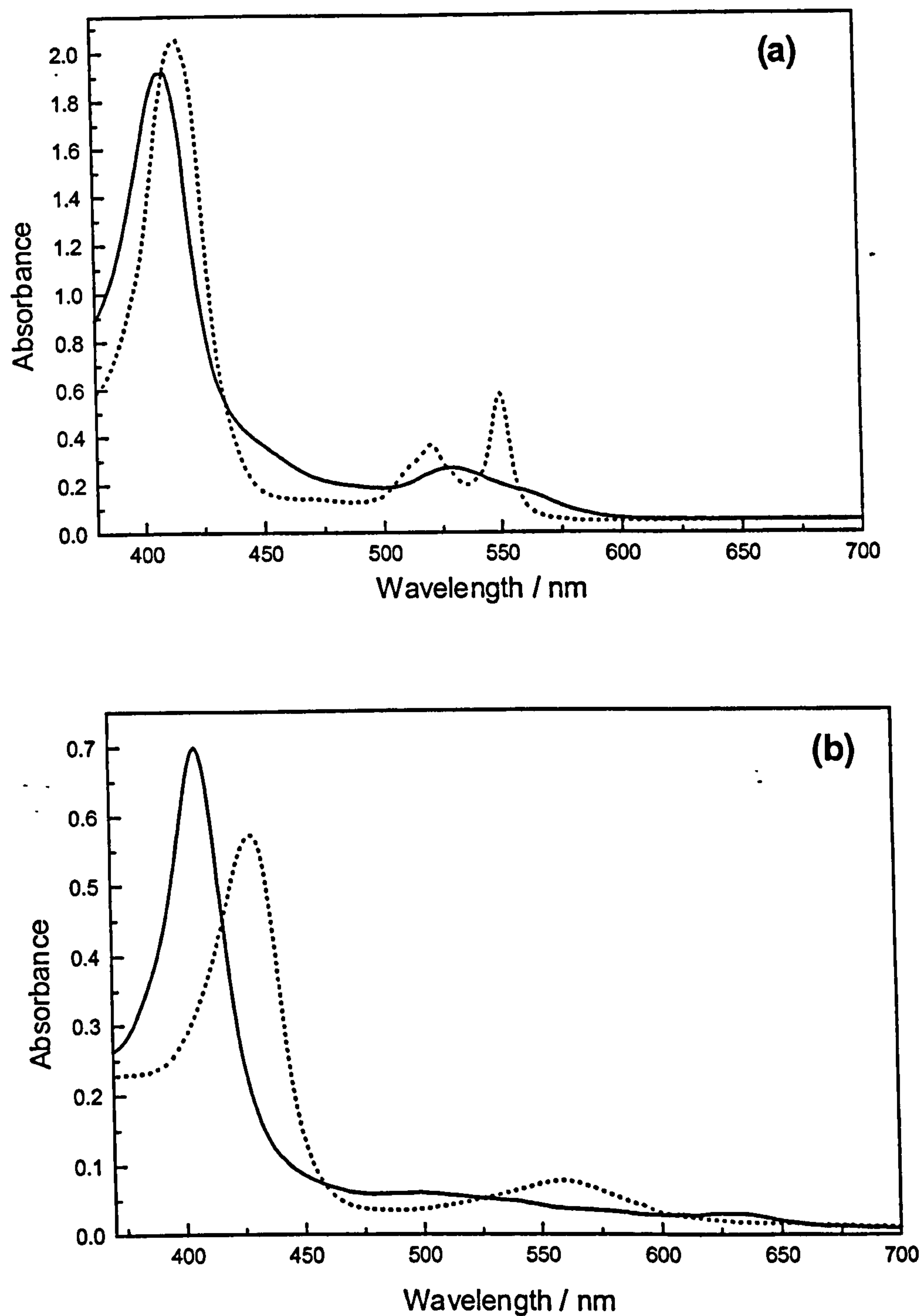


Figure 5.2: Absorption spectra of (a) Cyt-*c*/TiO₂ and (b) Hb/TiO₂ films before (—) and after (·····) steady state UV-illumination of the film at 337nm, demonstrating the photochemical reduction of Fe(III) Cyt-*c* to Fe(II) Cyt-*c*, and of met Hb Fe(III) to deoxy Hb Fe(II) driven by band gap excitation of the TiO₂ film.

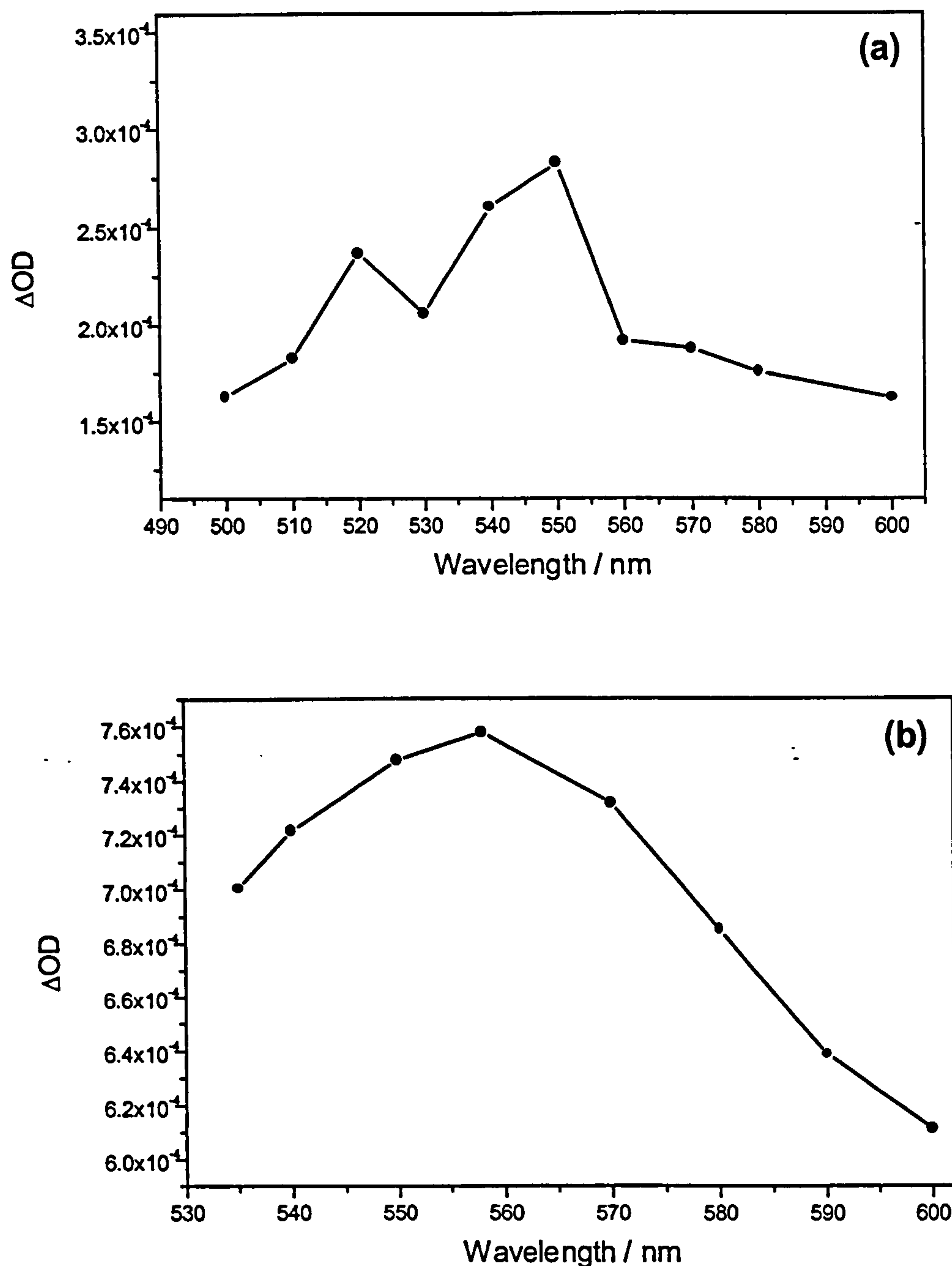


Figure 5.3: Transient absorption difference spectra of (a) Fe Cyt-*c*/TiO₂ and (b) Hb/TiO₂ films determined 1 μ s following band gap laser excitation (337 nm). Both spectra show the absorption increases characteristic of heme reduction for both proteins, demonstrating heme photoreduction in $< 1 \mu$ s. These features are superimposed upon a broad absorption increase assigned photogenerated electrons/holes in the TiO₂ film.

Both spectra show the characteristic absorption changes associated with heme reduction, indicating significant heme photoreduction occurs in $\leq 1 \mu\text{s}$. Control experiments with each protein in solution resulted in no detectable absorption changes on this timescale. Further control experiments on TiO_2 films in the absence of adsorbed protein gave rise to a broad, featureless absorption increase (see figure 5.4) which can be seen superimposed on the absorption changes associated with heme reduction shown in figure 5.3. This broad absorption increase is assigned to absorption by conduction band electrons and/or valence band holes in the TiO_2 film.

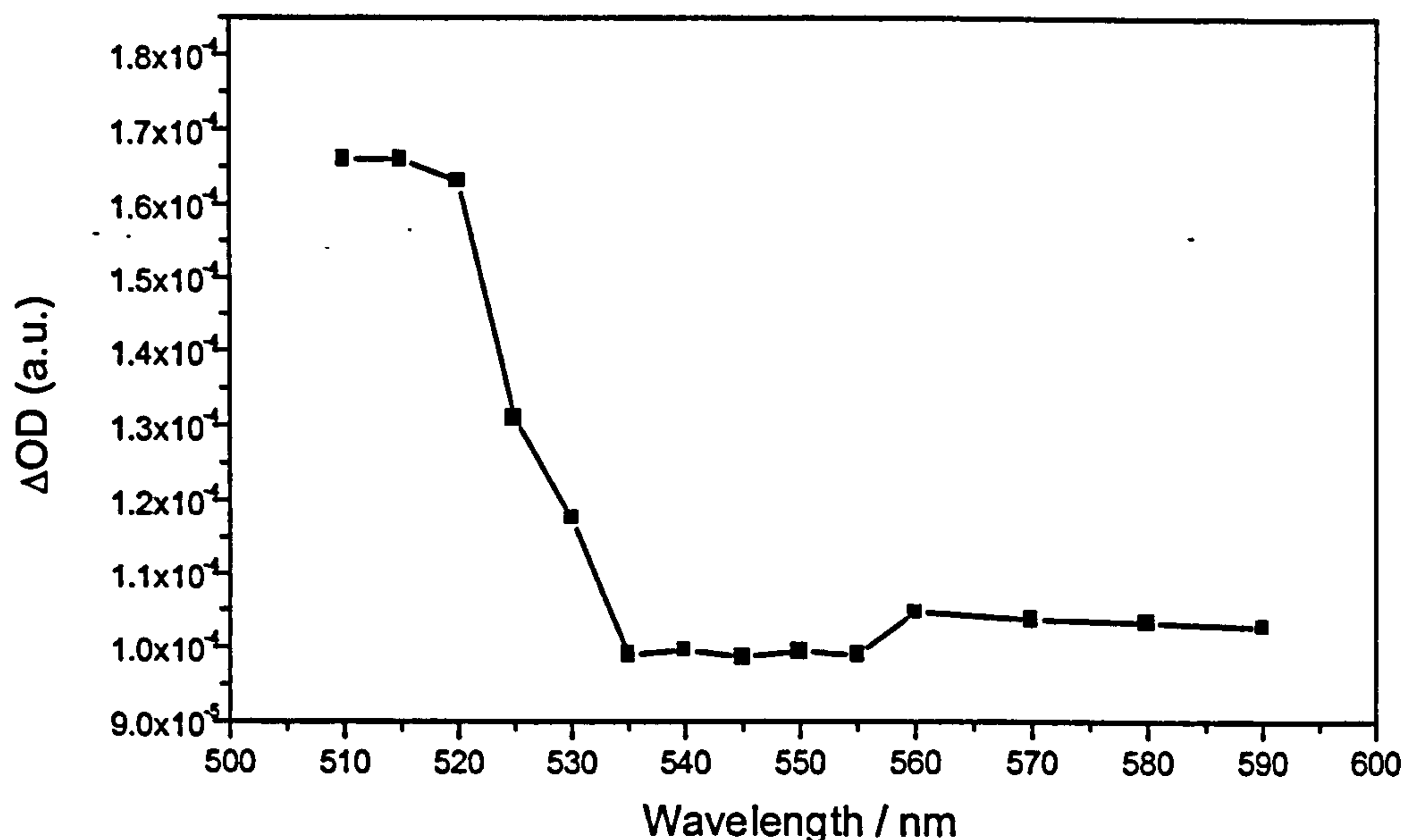


Figure 5.4: Transient absorption spectra of a blank TiO_2 film determined $1 \mu\text{s}$ following band gap laser excitation (337 nm). The spectrum shows a featureless absorption increase assigned to photogenerated electrons/holes in the TiO_2 film.

Figure 5.5 shows that the pulsed band gap excitation of a blank TiO_2 film in the absence of adsorbed protein, produced the same transient kinetics at 550 and 530 nm. These kinetics are due to the absorption by conduction band electrons and/or valence band holes in the TiO_2 film.

In order to measure the transient kinetics of Cyt-*c* reduction or oxidation the 530 nm signal was subtracted from the 550 nm signal. The 550 nm signal is a reduction peak of Cyt-*c* and an absorption increase or decrease should be observed when the protein is reduced or oxidised respectively. On the other hand any absorption change observed at 530 nm is due to absorption changes associated with charge carriers in the TiO_2 film. Therefore the signal obtained after subtraction correlates directly to absorption changes due to the protein and not the nanoporous TiO_2 film (Topoglidis et al., 2000).

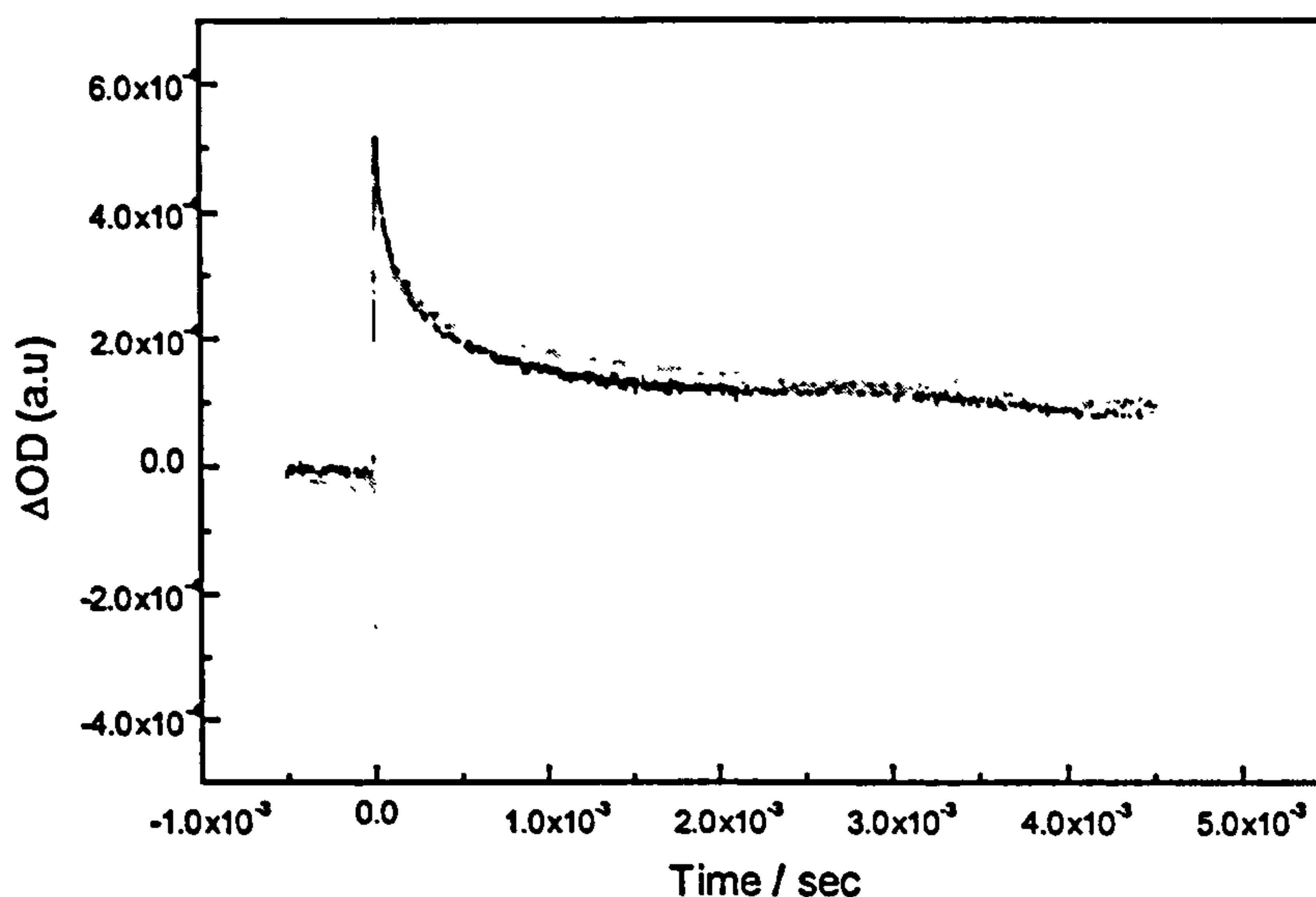


Figure 5.5: Transient absorption kinetics following band gap laser excitation (337 nm) of a blank TiO_2 film. The kinetics shown are the absorption changes observed at 550 and 535 nm.

Figure 5.6 shows the kinetics of photoreduction and photooxidation respectively of Cyt-*c* immobilised on TiO₂ films observed following pulsed 337 nm laser excitation.

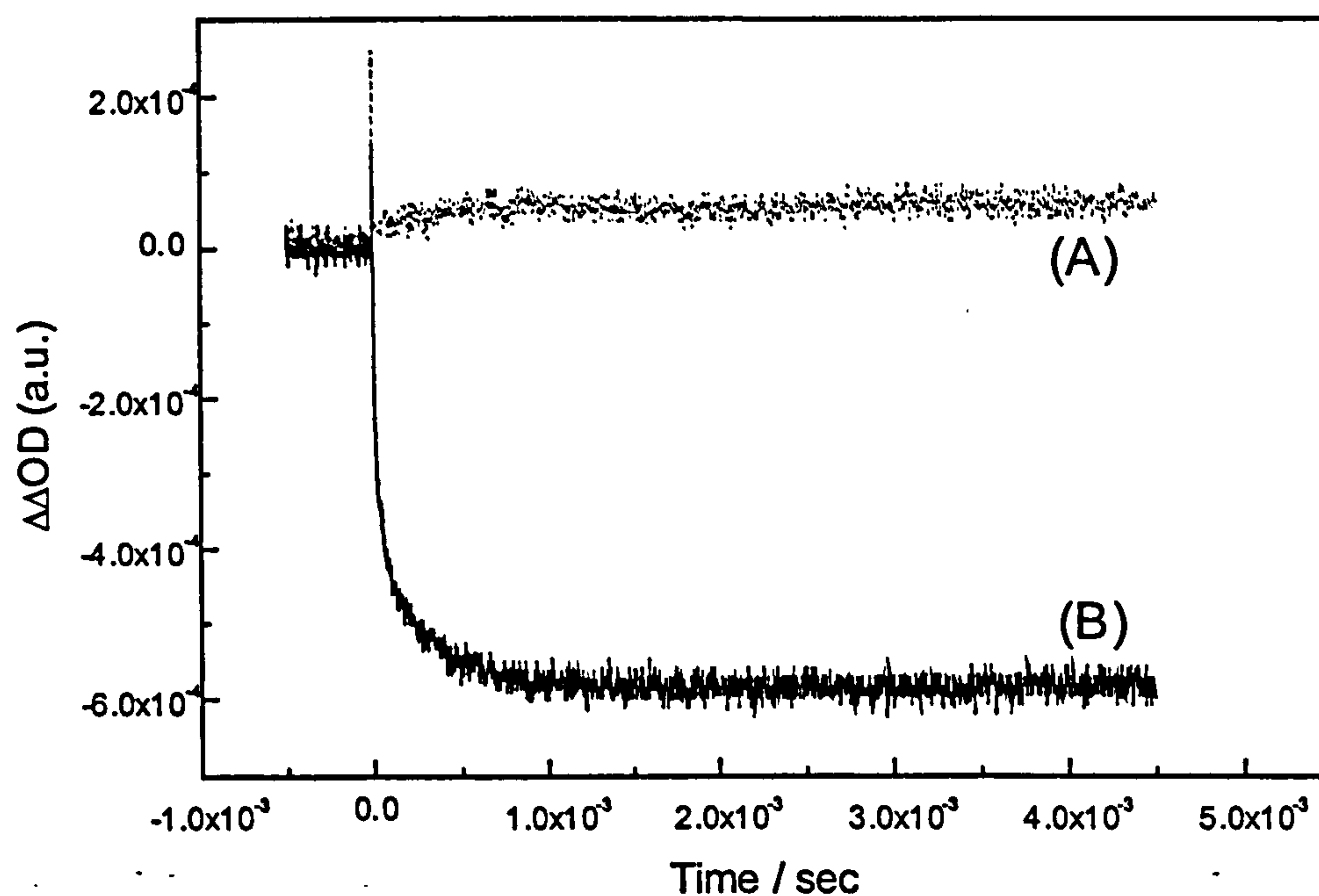


Figure 5.6: Transient absorption kinetics following band gap laser excitation (337 nm) of a Cyt-*c*/TiO₂ film. The kinetics are shown as the difference between the absorption changes observed at 550 and 535 nm in order to subtract absorption changes associated with electrons / holes in the TiO₂ film. A positive increase in the signal corresponds to net heme reduction. Data were collected with (A) the heme preoxidised (Fe(III) Cyt-*c*) resulting in net photoreduction, and (B) the heme prereduced (Fe(II) Cyt-*c*), resulting in net photooxidation.

Photoreduction kinetics were obtained on an initially Fe(III) Cyt-*c*/TiO₂, corresponding to the resting state in the dark. Reoxidation of the heme between laser pulses was achieved by the addition of 3 mM potassium ferricyanide. Photooxidation kinetics were obtained by pre-reducing the Cyt-*c* to Fe(II) by prolonged exposure to the laser excitation pulses (10-20 pulses) in the absence of ferricyanide. Data are shown as the difference between the absorption changes observed at 550 and 530 nm in order to subtract absorption changes associated with charge carriers in the TiO₂ film. The kinetics are at least biphasic with a fast phase ($< 1 \mu\text{s}$), and a slower phase with exponential lifetimes of 200 μs and 170 μs for photoreduction and photooxidation respectively (Topoglidis et al., 2000). Comparison of the magnitudes of the absorption changes with the intensity of the incident laser pulses (0.3 mJcm⁻²) indicates a quantum yield for heme oxidation per incident photon of ~5 %.

The cause of the observed biphasic reduction and oxidation kinetics is unclear. It is possible that the fast phase results from strongly bound proteins, whilst the slower phase results from proteins localised within the film pores but not strongly bound. We further note that, in contrast to cyclic voltammetry studies of protein/ electrode electron transfer, the light driven redox reactions reported here are associated with significant negative free energy changes.

5.4 Conclusions

The nanoporous TiO_2 films allow facile spectroscopic study of protein/electrode electron transfer processes. Furthermore, the observation that the redox chemistry of proteins adsorbed to the TiO_2 film may be driven optically is of interest for future spectroscopic studies of intraprotein electron transfer dynamics, and for photocatalytic applications.

CHAPTER 6

DEVELOPMENT OF OPTICAL BIOSENSORS BASED ON THE IMMOBILISATION OF PROTEINS ON NANOPOROUS TiO₂ FILMS

6.1 Introduction

Biosensor design and technology have prospered in the past two decades, and numerous well-defined approaches for effective sensor systems have been reported. Despite the success of many of these methods in the laboratory, however, only a few biosensors have been commercialised. Currently biosensors are being used primarily in clinical testing (e.g. monitoring blood glucose levels) but the potential application of biosensors as in situ probes for industrial environmental monitoring and control is very promising.

Proteins are frequently chosen as the molecular recognition molecules in the fabrication of optical and electrochemical biosensors. These biomolecules have the capability to bind target analytes with high specificity and fast reaction rates (Guilblaut et al., 1984). The basis of an optical biosensor occurs when the binding of a target analyte is accompanied by a unique change in the optical spectroscopic characteristics of the biomolecule. In order to utilise the desirable specificity properties of biological recognition molecules for sensing, a large amount of research has focused on methods of immobilisation, which achieve a high protein loading and do not interfere with the activity of the biomolecules.

6.1.2 Optical Biosensor based on the Encapsulation of Hb in Sol-Gels

A number of studies have focused on the encapsulation of proteins, as the biological recognition molecules, in sol-gel derived silicate matrices for the development of optical biosensors. Hb and Myoglobin (Mb) have been previously encapsulated in sol-gels to obtain semi-quantitative information about their biosensing capability and their reversible nature for the successful detection of dissolved gaseous NO, CO and O₂ using UV/VIS spectrophotometry (Ellerby et al., 1992, Blyth et al., 1995 and Chung et al., 1995, Lan et al., 1999). It has been shown that the encapsulation of these metalloproteins in sol-gels has no deleterious effects to their structure or activity and these proteins successfully retain their functional characteristics (i.e. their binding of oxygen and the formation of nitrosyl or carbonyl complexes with NO and CO respectively) (Blyth et al., 1995). The binding of CO and NO to the sol-gel encapsulated Hb was reversible. The carbonyl or nitrosyl (FeII) derivatives of each protein were reversed by oxidising them with excess potassium ferricyanide to produce the met (FeIII) form of each protein. On addition of sodium dithionite, the carboxy (FeII) derivative of each protein was obtained and the same cycle was repeated several times.

The biggest disadvantage of this biosensor was that the reactions took up to 2 hours to reverse making dynamic measurements impossible. In addition, the lack of electronic conduction in silica gels means that the redox state of the encapsulated proteins cannot be controlled electrochemically. The use of dithionite and potassium ferricyanide were necessary in order to reduce and re-oxidise the encapsulated metalloproteins. Recent advances of sol-gel methodologies of other materials have provided access to different gel matrices and the conducting properties of (V₂O₅) gels have attracted considerable attention (Glezer et al., 1993). Electronic conduction in

V_2O_5 gels does take place but the dark green-brown colours of these gels render them optically non-transparent and preclude the use of optical methods for signal processing.

6.1.3 The use of Hb/TiO₂ films for the optical detection of CO

In this chapter nanoporous TiO₂ films would be used as an alternative way to the sol-gel method to develop optical biosensors. The nanoporous TiO₂ films could provide an ideal immobilisation surface for the development of optical biosensors based on the reactivity of the immobilised proteins because they are optically transparent, have a high surface area, exhibit good electrical conductivity and can be prepared by low cost screen printing technologies.

In chapters 3 and 4, it was shown that Hb was immobilised successfully on TiO₂ films by physical adsorption, a high protein loading was achieved and the adsorbed Hb retained also its electrochemical activity. The heme group of Hb possesses open coordination sites, which can bind small-unsaturated exogenous molecules using oxygen or carbon monoxide electron lone pairs (Antonini et al., 1971). The reaction of Hb with oxygen or CO molecules is accompanied by specific distinct changes occurring in the absorption spectrum. The TiO₂ films are optically transparent and therefore provide an ideal surface for the development of an optical sensor based on the reactivity, analyte specificity and optical characteristics of the immobilised proteins. Therefore the reaction between the immobilised Hb and its specific analytes will initiate an optical response directly correlated to the substrate concentration which would be easily recorded using absorption spectroscopy.

Therefore, the TiO₂ films could provide an efficient biosensing design where the binding of the recognition molecule would be stable and its motion would be restricted, while the flow of analyte molecules (O₂ or CO) to the reactive site of Hb will be allowed through the porous structure of the TiO₂ nanoparticles.

6.2 Experimental Methods

6.2.1 Protein immobilisation

A solution of Hb (20 µM) was prepared in sodium dihydrogen phosphate buffer and the pH was adjusted to a value of 8 using NaOH. The solution was then deoxygenated by bubbling with Argon to prevent oxidation of the CO species. Immobilisation of Hb on the nanoporous TiO₂ films was achieved as described in chapters 2 and 3. The films were left in protein solution for at least 10 days.

6.2.2 Preparation of the CO solution

The CO gas cylinder was obtained from BOC gases. A 1 mM aqueous stock solution of saturated CO was prepared anaerobically by bubbling CO gas (1 atmosphere) through 2 ml of 10 mM NaH_2PO_4 buffer solution of pH 7 for 1 hour (Kaye et al., 1966).

6.2.3 Optical biosensing titrations

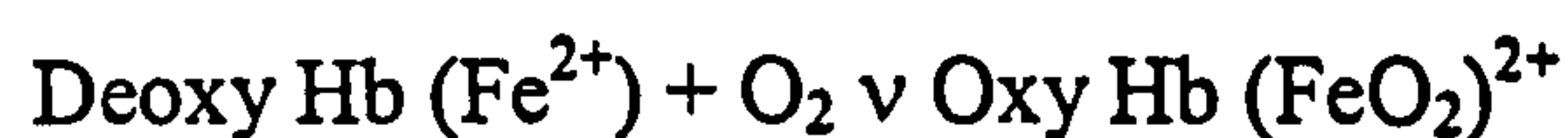
The immobilised metHb on the nanoporous TiO_2 film was electrochemically reduced to deoxyHb in the homebuilt 3 electrode electrochemical cell as described in chapters 2 and 4. The electrolyte solution used was 3 ml of 10 mM NaH_2PO_4 of pH 7, which was deoxygenated with argon prior to any optical/electrochemical measurements to prevent oxidation of the CO species. CO binding was monitored by absorption spectra collected before and after the addition of a saturated solution of CO to the deoxyHb/ TiO_2 film. The CO solution was titrated by a GSE microsyringe into the electrochemically reduced deoxyHb/ TiO_2 film in the electrochemical cell. Absorption spectra were taken 4 minutes after each CO addition. All experiments were carried out at room temperature.

6.3 Results and Discussion

6.3.1 Optical Biosensing Activity of immobilised Hb

The Hb high affinity for O₂ coupled with the changes in visible absorption provide an opportunity to develop a dioxygen sensor.

The Hb/TiO₂ film was first confirmed to be in the met form from its visible absorption spectrum. Only the deoxyFe(II)Hb is able to bind O₂ successfully and therefore met Fe(III)Hb was electrochemically reduced to the deoxy form by the application of a -0.7 V potential. Oxy hemoglobin was then generated by dropping the potential to 0 V and by passing O₂ through the electrochemical cell for 1 minute. The chemical and physical changes are shown below:



Oxygen binds to the heme iron atom(s) of deoxyHb and conformational changes on their structures occur. After the binding of O₂ the sixth coordination site is occupied by oxygen. The visible absorption spectra of metHb, deoxyHb and oxyHb immobilised on nanoporous TiO₂ films are shown in figure 6.1. Following the application of a -0.7 V bias and the subsequent bubbling with O₂, the Soret band at 428 nm of the deoxy Hb shifted to 415 nm and the broad band at 558 nm disappeared and was replaced by two new bands at 541 and 576 nm. These absorption bands are identical to those of the corresponding oxyHb derivative in solution (Antonini et al., 1981) and indicating that immobilisation of Hb on the TiO₂ films does not change the structure of the protein and retains its sensitivity for O₂.

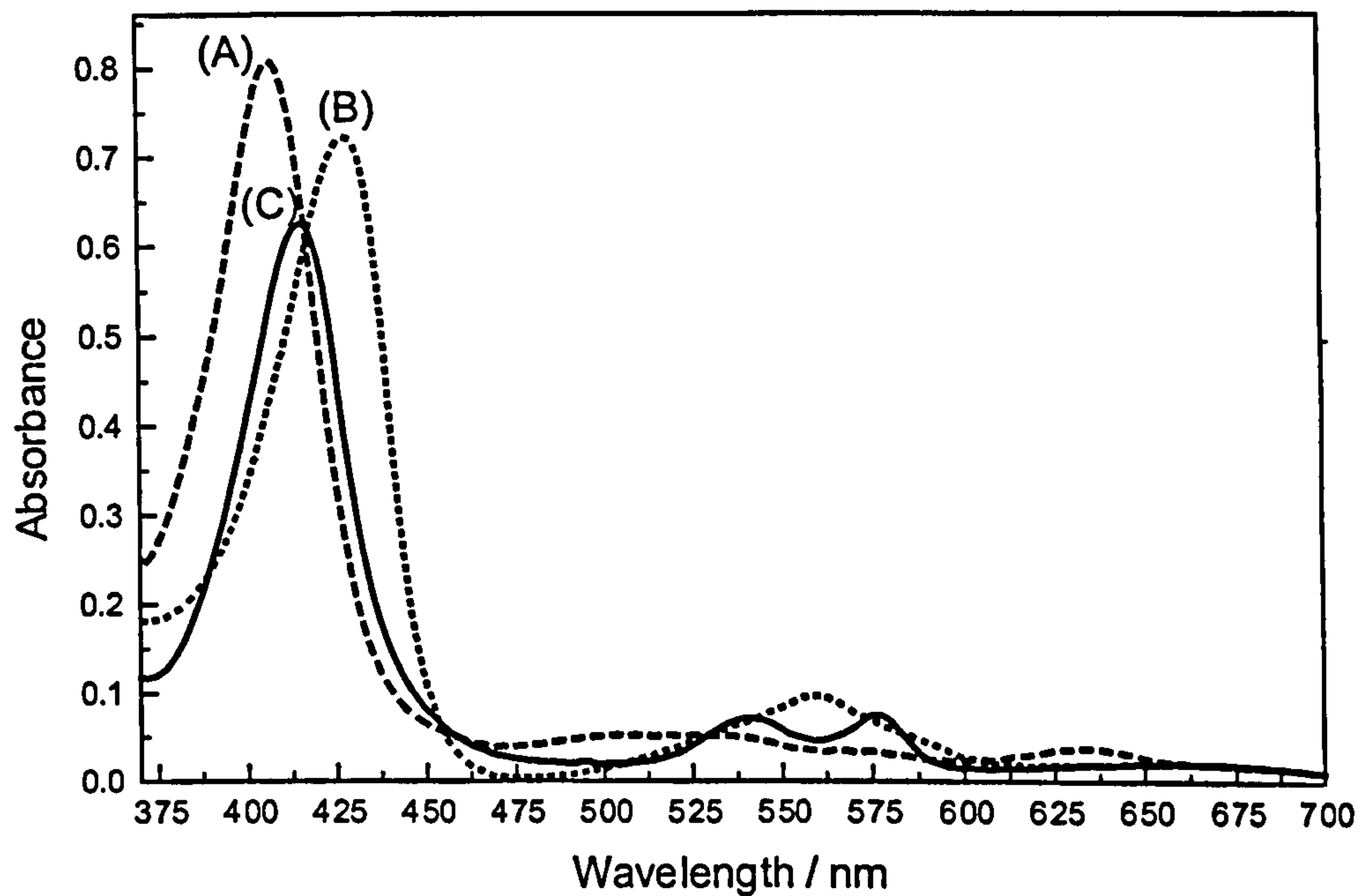


Figure 6.1: Absorption spectra of (A): metHb (B): deoxy Fe(II) Hb, (C) oxyHb(II) Hb immobilised on a nanoporous TiO₂ film.

Furthermore, by applying increasing positive bias and/or bubbling argon (to remove the O₂ from the electrochemical cell) for 15-25 min it was possible to convert the immobilised oxy Fe(II)Hb back to its oxidised met form. The cycle was repeated again on the regenerated film successfully, with only minor desorption (5-8%) of the immobilised Hb. This shows reversibility of the binding and that the Hb/TiO₂ film can be reused for subsequent determinations.

In this thesis the aim is to show that the immobilised Hb on a TiO₂ film is able to retain its high affinity for O₂ and not to quantify the optical changes for application as biosensor elements. However, the viability of this approach was used for the sensing of CO in aqueous solution by optical spectroscopy of a Hb/TiO₂ film because the binding affinity of Hb is greater for CO than for O₂ (Antonini et al., 1971).

As for O_2 , carbon monoxide will only form a stable complex with Hb when the iron is in the deoxy Fe(II) state (Antonini et al., 1971). The CO derivative of hemoglobin, carbonyl Fe(II)Hb, is a particularly stable derivative. CO binds much more tightly than oxygen to heme iron atoms. The preferential binding of CO to heme iron is largely responsible for the asphyxiation that results from CO poisoning.

The immobilised Hb was initially reduced to its Fe(II), deoxy, state by the application of -0.7 V to the TiO_2 film, as for figure 6.1. Subsequently the addition of a saturating amount of aqueous CO resulted in the characteristic heme absorption changes associated with CO binding, as shown in figure 6.2.

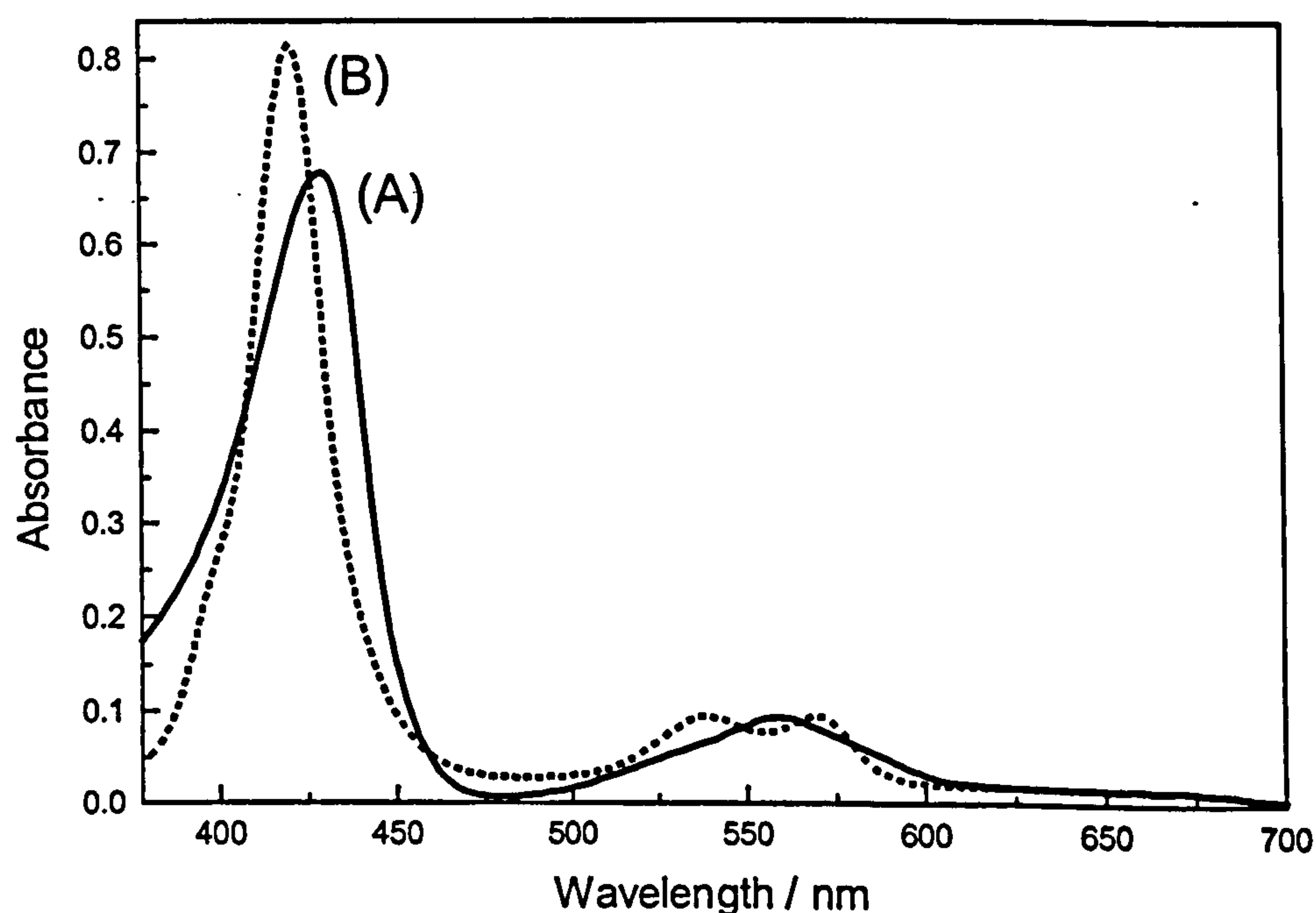


Figure 6.2: Absorption spectra of (A): deoxy Fe(II) Hb, (B) carbonyl Fe(II) Hb immobilised on a nanoporous TiO_2 film.

Binding of CO to the Fe(II) Hb/TiO₂ film results in a shift in the heme Soret absorption maximum from 428 to 420 nm, accompanied by the appearance of α and β absorption bands at 540 and 568 nm, in good agreement with solution phase studies of CO binding. These absorption changes could be titrated by the progressive addition of small volumes of 1 mM CO solution to the photoelectrochemical cell, as illustrated in figure 6.3.

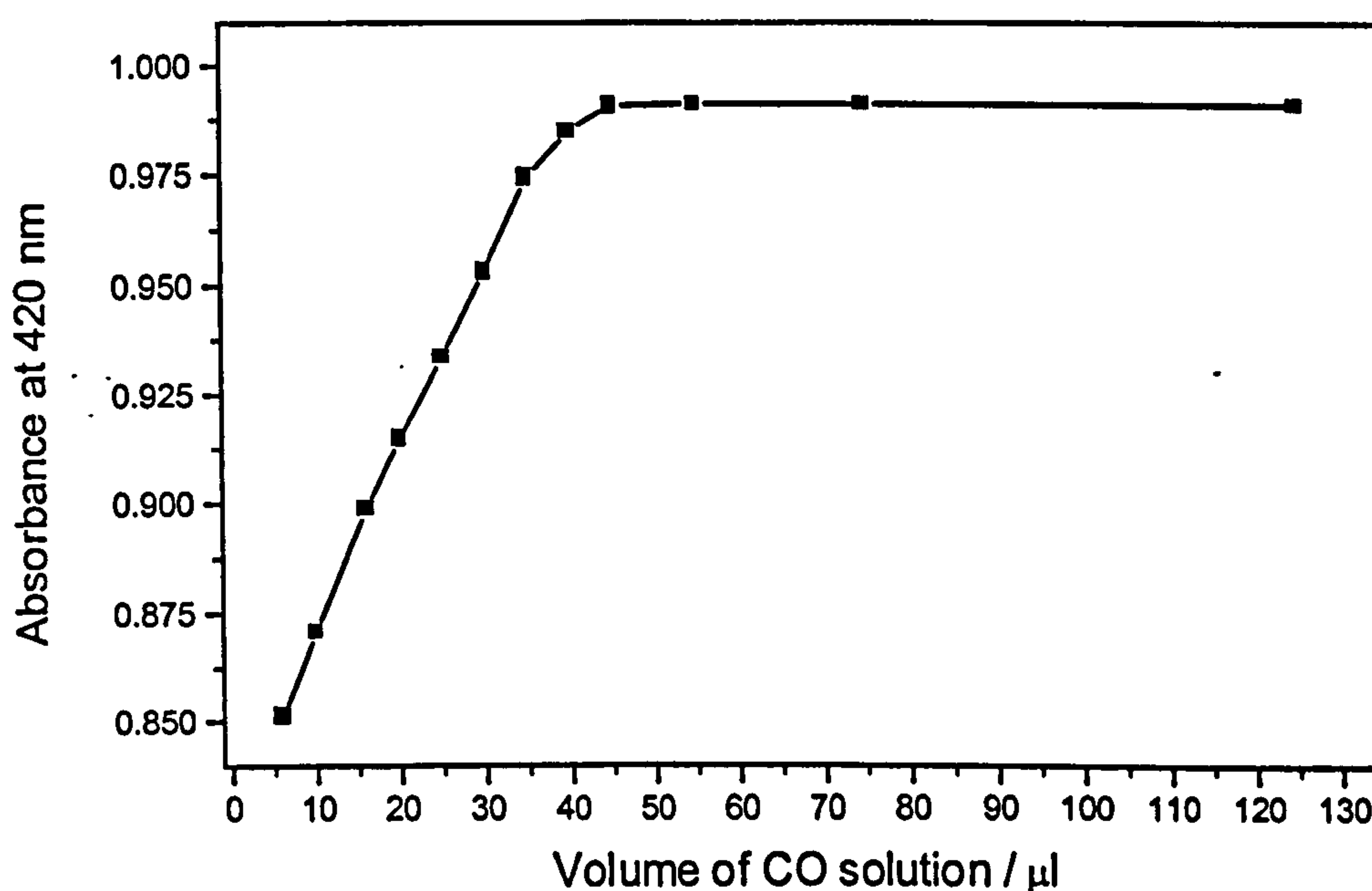


Figure 6.3: Carbon monoxide binding curve of Hb immobilised on a TiO₂ film. Deoxy Fe(II) Hb titrated against additions of saturated CO solution. The increasing absorption intensity was monitored at 420 nm.

A linear increase in absorbance at 420 nm is observed as a function of CO added to the cell, saturating after the addition of ~ 40 nmoles CO to the 3.5 ml buffer solution in the cell (corresponding to 11 μ Molar). The detection sensitivity was approximately 5 nMoles CO. After each addition of CO solution, the absorbance change was complete within 4 minutes. After the end of the titration the CO was unbound from the immobilised Hb by the application of a positive potential (+0.4 V) resulting in the oxidation of the carboxy Fe(II) Hb to met Fe(III) Hb. Following CO desorption, the Hb can be rereduced by the application of -0.7 V, and reused for the determination of CO concentrations. The oxidation process could be further accelerated by bubbling the solution with oxygen whilst applying +0.4 V.

We note that the procedure as detailed here (Topoglidis et al., 2000) is unlikely to be directly applicable to the development of a technological sensor. For example each complete cycle resulted in 5-6 % protein desorption, limiting the repeated use of a single film to 3-4 separate CO binding titrations. Moreover the sensitivity range for CO detection is probably insufficient for such applications. However the results do demonstrate the viability of the approach. Studies are currently under way to optimise the film properties, protein binding and detection strategies in order to address such issues.

6.4 Conclusions

The structure and activity of Hb are preserved after its adsorption on nanoporous TiO₂ films. Hb/TiO₂ films show characteristics that make them excellent sensors for dissolved O₂ and CO. These properties arise from a combination of the O₂ or CO binding property of the deoxyHb and the optical transparency and porous structure of the TiO₂ films. The Hb/TiO₂ films have been shown to exhibit a sensitive optical response after binding to O₂ or CO, and it is established within 1-2 minutes. The procedures and equipment used is relatively simple and cheap. They have also the advantage over the sol gels that they are not only optically transparent but also show electronic conduction. Therefore, the prospects are excellent for the use of these films in the development of an optical biosensor for the quantitative and reversible detection of dissolved O₂ and CO in aqueous solutions.

6.5 The use of TiO₂ Films in the Development of a Fluorescence / Optical Biosensor

6.5.1 Motivation

The development of this biosensor involves a well-characterised naturally occurring protein with the desired ligand specificity, the discovery of a suitable signal and the construction of a detector adapted to the protein in question (Gilardi et al., 1994, 1997). In this type of biosensor, sensing is provided by the interaction between a biological macromolecule, IANBD-labelled maltose binding protein (S337C-NBD), and its ligand, (e.g. maltose). Detection occurs as the fluorescence intensity S337C-NBD exhibits a strong increase following ligand binding. The fluorophore IANBD (figure 6.4) was chosen properly to label the MBP so that the ligand induced changes in the protein will yield quantifiable changes in the fluorescence from the IANBD.

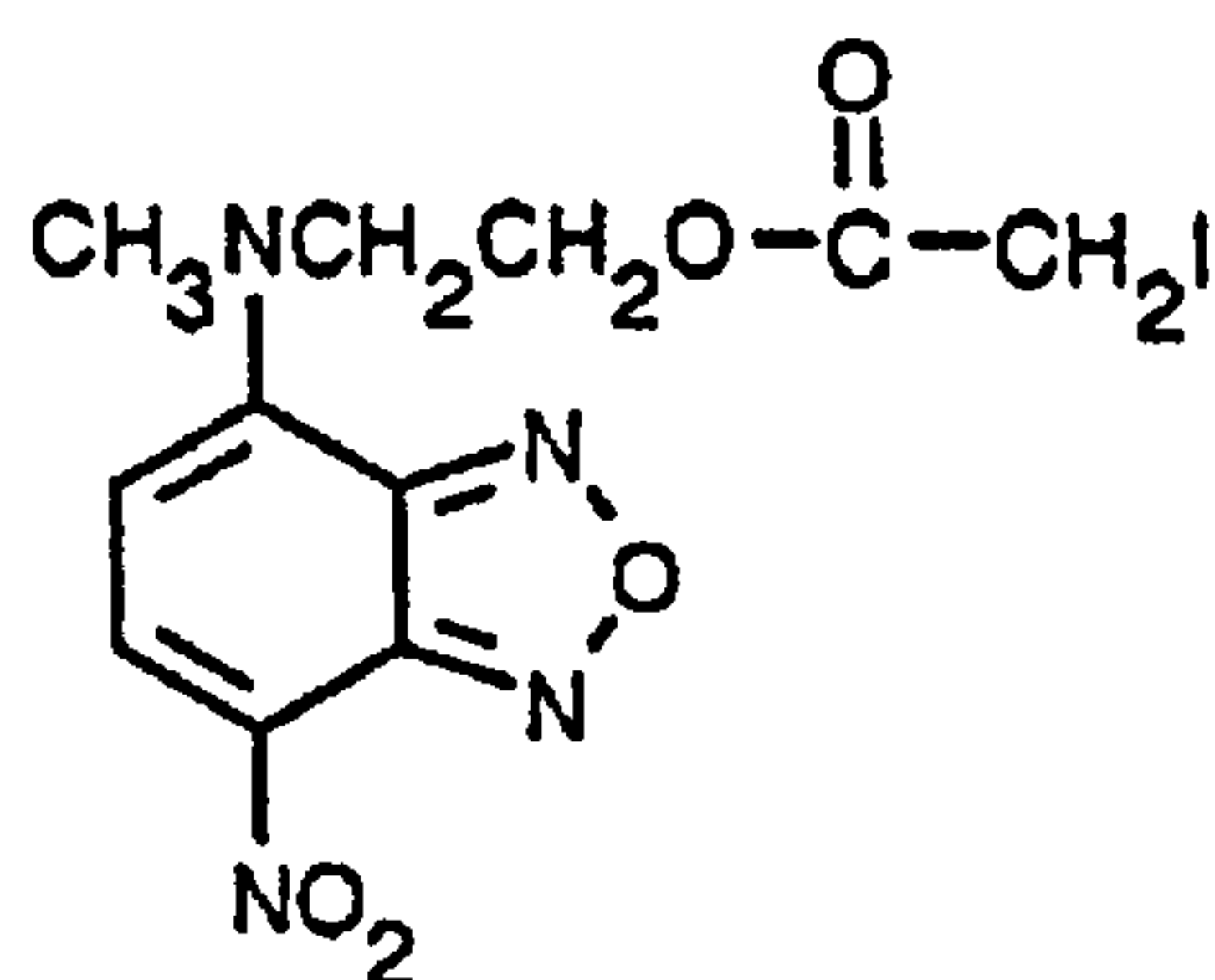


Figure 6.4: Structure of N,N'-dimethyl-N-(iodoacetyl)-N'-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine (IANBD amide): Molecular structure: C₁₂H₁₄IN₅O₄

However to use a biorecognition element (S337C-NBD) as part of a biosensor it must be immobilised upon a solid surface and it becomes critical to know how the fluorophore accessibility within the MBP is affected by immobilisation. S337C-NBD has not been incorporated into an analytical device as yet and therefore TiO₂ films could provide the solid surface for the development of a fluorescence/optical reagentless biosensor based on S337C-NBD and its ligand binding.

6.5.2 Maltose binding protein

The MBP is a member of the family of the periplasmic ligand binding proteins of gram-negative bacteria that form part of the chemotaxis and nutrient-uptake systems (Furlong et al., 1988) and are specific for a wide variety of ligands. MBP is specific for its ligands, maltose and β -cyclodextrin (Gilardi et al., 1994, 1997, and Marvin et al., 1997).

MBP has been extensively characterised by genetic, biochemical and structural studies. MBP is a monomeric protein with a molecular weight of 40,6 kDa and the pre-protein is 396 amino acid residues in size (Spurlino et al., 1991). It is composed of a single chain that folds into two domains connected by a hinge region (Sharff et al., 1992). The ligand-binding site is located in the interface between the two domains (see figure 6.5). Ligand-binding is accompanied by a large conformational change, in which the two domains close around the ligand, involving a bending and twisting motion around the hinge region, thus “trapping” the ligand inside (Gilardi et al., 1994 and Marvin et al., 1997) (see Figure 6.6).

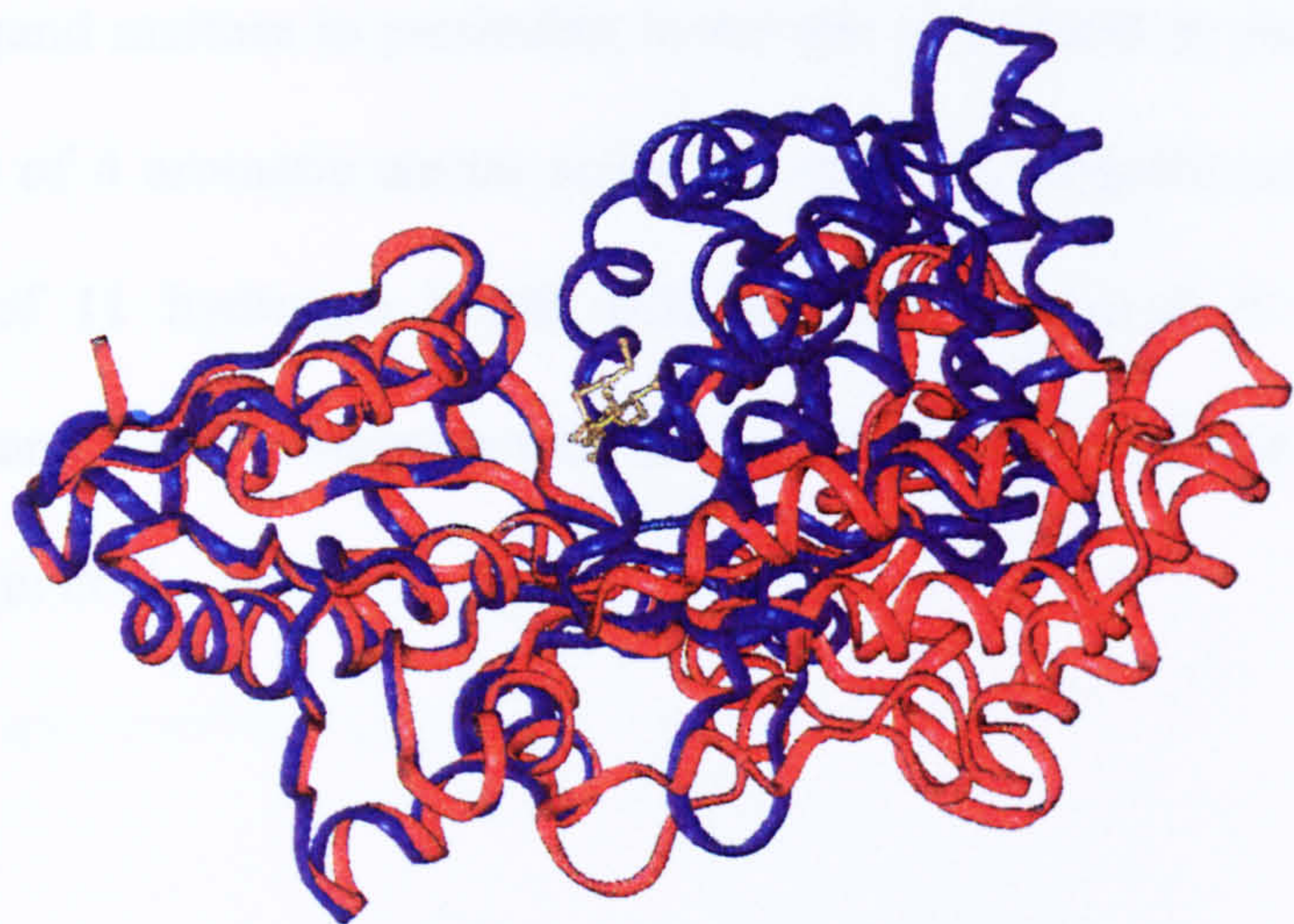


Figure 6.5: Structure of the maltose binding protein. The Ca backbone of the ligand free (open) protein, red, is overlaid with that of the maltose bound (closed) protein, blue. The maltose is shown in yellow.

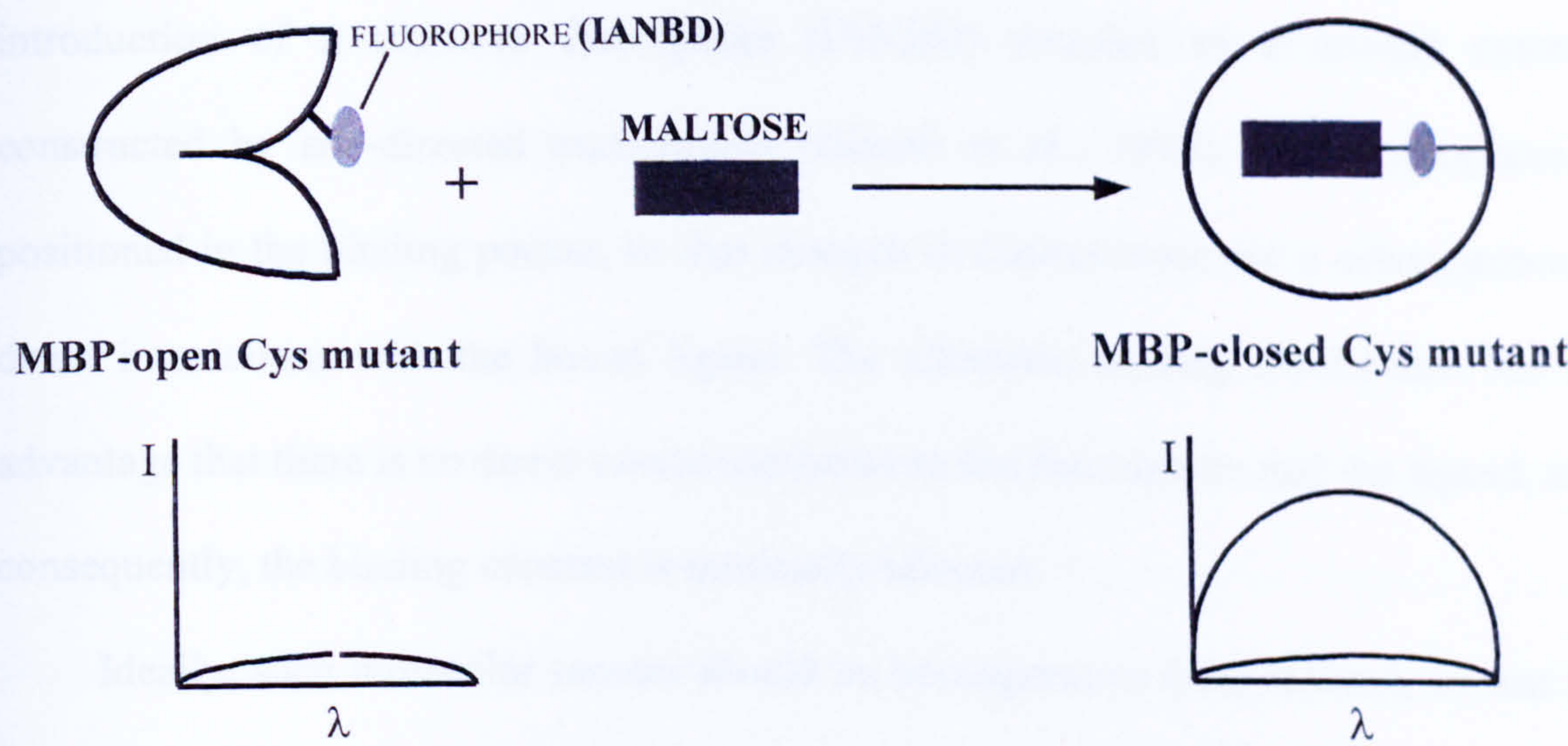


Figure 6.6: Schematic drawing showing the effect of ligand binding on the structure of the maltose binding protein and its consequences for the fluorescence of a site specifically introduced fluorophore.

The ligand maltose in particular, is thought to be held in place by the hydrophobic interactions of 4 aromatic amino acids and the C-C skeleton of the sugar, and by the formation of 11 hydrogen bonds with the side chain of 6 charged residues, one tryptophan and water molecules with the hydroxyl groups of the maltose (Gilardi et al., 1997, Marvin et al., 1997).

6.5.3 Biosensor Design

This system has been exploited to introduce single fluorophores that respond to ligand binding in the MBP. The transducing element was provided by the site-specific introduction of a sensitive fluorophore (IANBD) coupled to a unique cysteine constructed by site-directed mutagenesis (Gilardi et al., 1994). The fluorophore is positioned in the binding pocket, so that changes in fluorescence are a consequence of direct interactions with the bound ligand. The allosteric sensing mechanism has the advantage that there is no direct interaction between the fluorophore and the ligand, and, consequently, the binding constant is minimally affected.

Ideally, such molecular sensors should be homogeneous (reagentless), so that the measurement does not require a change in the composition of the sensor, unlike heterogeneous systems (protein based systems), which consume the analyte.

Among the molecular properties resulting from the conformational alteration, the fluorescence change is the best studied (Gilardi et al., 1994, 1997). It is the strong increase in the fluorescence yield, occurring upon ligand binding, that is used to provide the starting point for the sensor development. Although there have been many kinds of

fluorescence biosensors developed, most of them based on reversible binding the assay usually involves a competition between labelled and unlabelled ligand for the receptor protein. One advantage of using S337C-NBD as the receptor is that because it shows a change in its fluorescence properties when ligand binds this means it can be used as a reagentless sensor. That is, it is the property of the receptor that is being measured rather than a competing ligand.

6.5.4 Immobilisation on TiO₂ films

In order to incorporate the S337C-NBD into a fluorescence/optical biosensor, it is necessary to immobilise the S337C-NBD on a solid surface. Immobilisation of S337C-NBD to a solid surface and investigation of its relative characteristics relative to the native S337C-NBD are crucial in the development of the biosensor. Many physical and chemical methods for immobilising proteins on solid surfaces, like adsorption and entrapment (see introduction) have been widely used in the past but problems, such as protein conformational changes affecting the functional activity and detachment of the protein resulted in a less sensitive and active protein showing short longevity. In this project the ability of the S337C-NBD to adsorb onto the surface of TiO₂ films was studied. The high surface area and optical transparency of the TiO₂ films suggested that these may be appropriate as inert substrates for the immobilisation of fluorophore labelled enzymes for bioanalytical applications (e.g. optical fluorescence sensors which take advantage of protein - ligand interactions). Then a series of experiments were carried out in order to find out if the S337C-NBD loses its affinity for its ligands, maltose and β -cyclodextrin, after it is adsorbed on a TiO₂ film.

6.6 Experimental Procedures

6.6.1 Preparation of S337C-NBD

The S337C MBP mutant was prepared by Nickolas Beaumont in the laboratory of Dr. Tony Cass at Imperial College, following the standard procedure of Gilardi et al., 1994. It was supplied in 10 mM potassium dihydrogen phosphate buffer solution of pH 7.0 and the protein concentration was of 0.5 mg ml⁻¹. The thiol reactive fluorophore, 4-[N-2-(iodoacetoxy) ethyl-N-methylamino]-7-nitrobenz-2-oxal, 3-diazole (IANBD ester) was used to specifically label the MBP, (S337C-NBD). Labelling conditions were optimised in terms of label/protein concentration and time following the published procedure of Gilardi et al., 1994. The fluorescence yield of the attached fluorophore exhibits a strong increase following binding of maltose and β -cyclodextrin. A 10 mM maltose and a 10 mM β -cyclodextrin stock solution were also supplied.

6.6.2 Immobilisation of S337C-NBD on TiO₂ films

The preparation of TiO₂ films was done as described in chapter one (section 2.2). The immobilisation of the labeled MBP (S337-NBD) was achieved by the immersion of a 1 cm² TiO₂ film in aqueous S337-NBD protein solution (0.025 mg/ml) at 4°C for 1-5 days. This is a very simple, well-established procedure (chapter 1, section 2.3). After the fifth day, the immobilised TiO₂ films were stored in protein free buffer solution for up to 10 days and this resulted in no detectable desorption of the protein from the film. These films were used in the subsequent experiments within 1 week.

6.6.3 Fluorescence measurements

All fluorescence measurements were made on a Perkin-Elmer Luminescence Spectrometer LS-50B controlled by a PC with FLWin Lab software at room temperature. The immobilised films were placed at a fixed angle in a 3-ml fluorescence cuvette, filled with 3 ml of 10 mM KH_2PO_4 pH 7. Fluorescence emission spectra were obtained by excitation of the S337C-NBD/ TiO_2 films at 478 nm. The slit chosen was 15 nm and the speed was 300 nm min⁻¹. A fluorescence emission spectrum was plotted, which measures the relative intensity of the fluorescence on the y-axis, across a range of wavelengths on the x-axis. The range of the x values are set so that they contain the wavelength at which the fluorophore (IANBD) emits.

In order to find out if the S337C-NBD retains its affinity for its ligands after immobilisation on a TiO_2 film, increasing concentrations of a 10 mM maltose stock solution were added in the cuvette, containing the immobilised film, and the mixture was stirred gently using the tip of a Gilson pipette. The fluorescence emission spectrum was monitored, and the procedure was repeated each time adding aliquots of maltose, until no further increase in fluorescence intensity was observed and the resulting peaks superimposed. The procedure was repeated using a 10 mM β -cyclodextrin stock solution instead of maltose. After each experiment the immobilised films were taken out, washed with protein free buffer solution in order to unbind and eliminate the ligand (maltose or β -cyclodextrin) and the procedure was repeated again. This if successful, would show that the increase in fluorescence intensity is fully reversible and allow the use of the immobilised films in more than one experiment.

6.7 Results and Discussion

6.7.1 Immobilisation of MBP on TiO₂ films

TiO₂ films are optically transparent and therefore the immobilisation of S337C-NBD was monitored using the fluorescence intensity spectra of the fluorophore labeled protein. Figure 6.7 shows the fluorescence emission spectrum of a S337C-NBD/TiO₂ film.

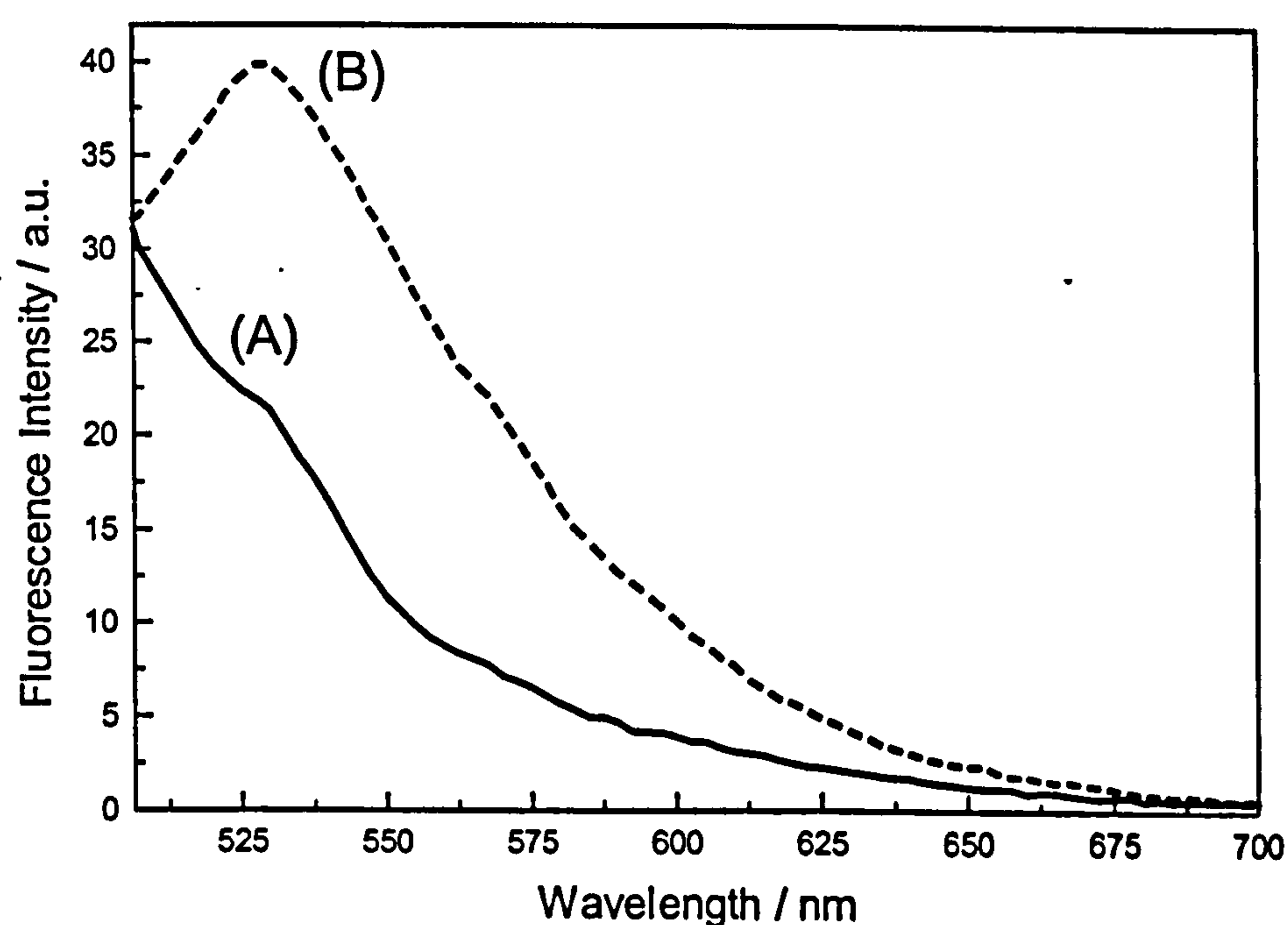


Figure 6.7: Fluorescence emission spectra of (A) a blank nanoporous TiO₂ film and (B) after the immobilisation of S337C-NBD upon its surface.

Immobilisation of S337C-NBD on the TiO₂ films reached a limiting amount after incubation of the film in the protein solution for 5 days at 4° C (approx. 50 % of the final adsorption was achieved within 1.5 days). Also shown in figure 6.7 is the fluorescence emission spectrum of the TiO₂ film prior to protein immobilisation, which does not interfere with the fluorescence emission of the protein. The binding of S337C-NBD on the surface of the film is quite strong because storage of the film in protein-free buffer solution for up to one week resulted in no detectable desorption of the protein from the surface of the film. S337C-NBD is the first non-redox protein that is immobilised on the TiO₂ films (Topoglidis et al., 1998).

The binding of S337C-NBD on the TiO₂ surface was confirmed by monitoring the IANBD label absorption at 500 nm. Comparison with dye binding studies suggests that S337C-NBD achieves a 20 ± 4 % coverage of the internal surface area of the film available for dye binding. The somewhat lower binding achieved with S337C-NBD compared with Cyt-*c* (60%, see chapter 1) is consistent with the larger dimensions, (diameter of S337C-NBD ~ 7 nm compared to 4 nm of Cyt-*c*) of this protein, which will prevent access to the smaller pores of the film (Topoglidis et al., 1998).

The S337C-NBD adsorption on the TiO₂ films is most probably based upon electrostatic interactions between the surface charge distribution of the protein and the TiO₂ film, like for Cyt-*c*, Mb and Hb. The positively charged basic amino acids, 44 lysines and arginines, which are spread all over the S337C-NBD, interact electrostatically with the negatively charged groups of the TiO₂ surface. It should be possible to further improve the stability of protein binding on the TiO₂ films by employing the covalent binding strategies already well established for the attachment of biomolecules to other metal oxide surfaces (see discussion of chapter 1).

6.7.2 Ligand binding on immobilised S337C-NBD

The functional integrity of the immobilised S337C-NBD protein was assessed by determination of its fluorescence yield as a function of substrate concentration. Previous solution studies have demonstrated that the fluorescence yield of the S337C-NBD increases as a function of maltose or β -cyclodextrin concentration (Gilardi et al., 1994, 1997 and Marvin et al., 1997). This fluorescence increase is specific to these sugars, the addition of, for example, sucrose to S337C-NBD in solution resulting in no detectable change in fluorescence yield. Figure 6.8 shows the corresponding data obtained for S337C-NBD immobilised upon the TiO₂ film.

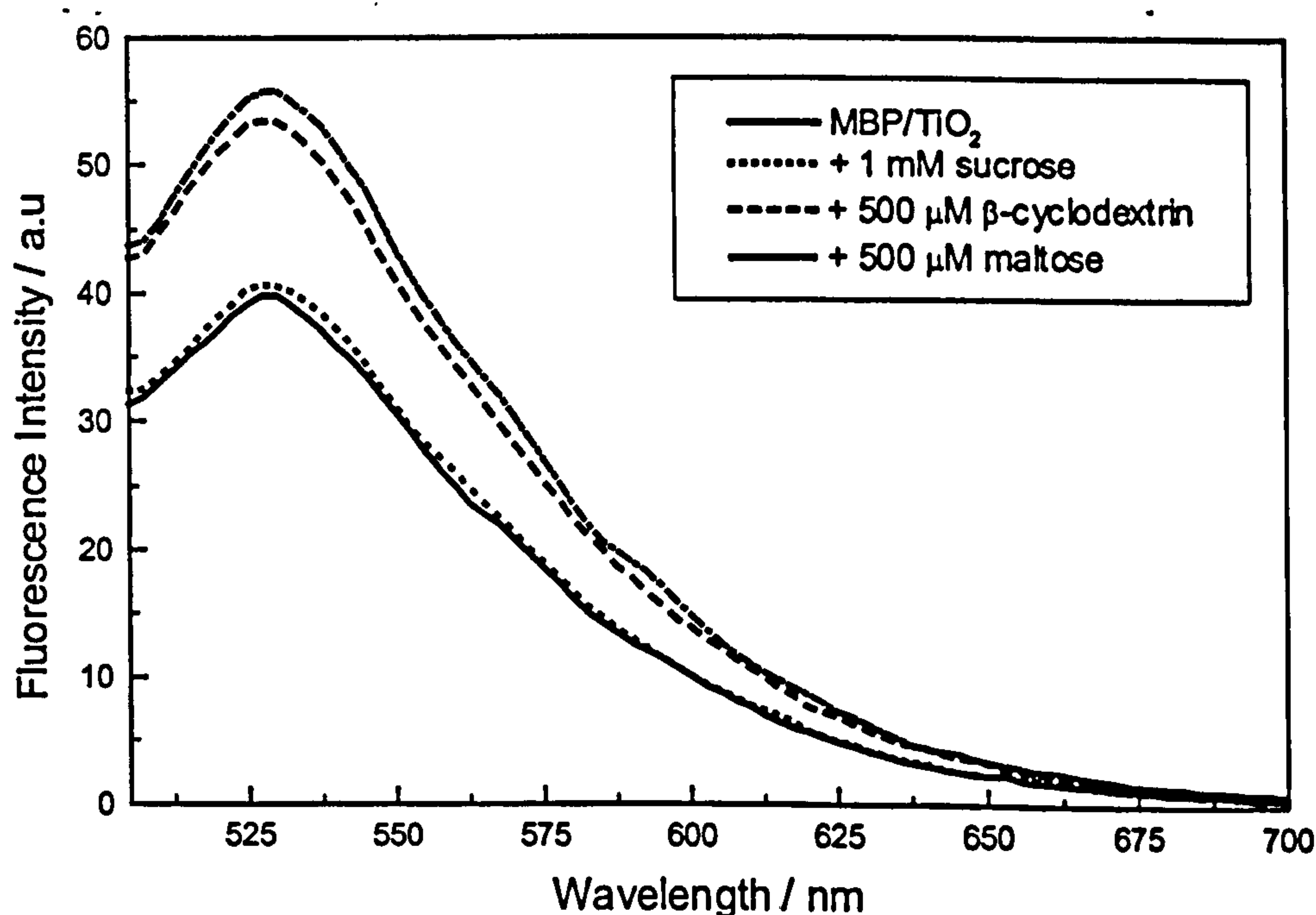


Figure 6.8: Fluorescence emission spectra of S337C-NBD immobilised upon a nanoporous TiO₂ film. Emission spectra were determined for the film immersed in protein free buffer solution before and after the additions of the sugars maltose, β -cyclodextrin and sucrose.

A large increase in fluorescence intensity of S337C-NBD/TiO₂ was observed following the addition of maltose (41 %) and β -cyclodextrin (35 %), whilst the intensity was essentially independent of the addition of sucrose. The reaction time between immobilised S337C-NBD and the adding concentrations of its two ligands was 2 minutes. The porous TiO₂ surface allows the immobilised S337C-NBD to react and bind its ligands, which are able to diffuse in the immobilised TiO₂ surface. The fluorescence increase was fully reversible; rinsing in buffer solution resulted in complete maltose unbinding with only minor S337C-NBD desorption (<5%).

It might be suggested that if desorption of the S337C-NBD is occurring to the extent of 5%, this immobilisation strategy will never work to make a viable sensor. The desorption of S337C-NBD was less than 5% in most of our experiments except once that exceeded 5%. However, in this project the aim was to show that TiO₂ films are a suitable solid surface for the immobilisation of viable biomolecules, which retain their properties and not the development of a sensor. Although our results are promising, there is a lot of work that should be done in order to use this method for the development of bioanalytical devices, such as biosensors (Topoglidis et al., 1998).

The addition of maltose results in a smaller percentage increase in fluorescence intensity than that observed for the labelled S337C-NBD in solution. Consistent with this observation, our initial studies indicate a binding constant for maltose to the immobilised S337C-NBD of $150 \pm 18 \mu\text{M}$, approximately 2.5 times higher than the K_D of S337C-NBD in solution (Gilardi et al., 1994). This can most probably be attributed to minor structural distortions or restrictions associated with the immobilisation. However, these distortions do not appear to have impaired the specificity of ligand binding by the S337C-NBD and would therefore be unlikely to limit the development of an optical biosensor based upon this system.

6.8 Conclusions

This is the first reported study of the immobilisation of S337C-NBD upon a solid surface, a nanoporous TiO_2 film. S337C-NBD has never been incorporated in the past into a bioanalytical device such as an optical/fluorescence biosensor that is suggested in this project. In this study, it was tried to understand the behaviour and performance of S337C-NBD immobilised on TiO_2 films. S337C-NBD was successfully adsorbed on the surface of the films using a very simple immobilisation strategy as for Cyt-*c*, Mb and Hb. The immobilised S337C-NBD retains its sensitivity and specificity for its ligands. Moreover the S337C-NBD/ TiO_2 films are optically transparent, can be readily and cheaply processed by screen printing technologies and have the ability to be reusable. This immobilisation strategy may therefore be of potential interest for the development of a fluorescence/optical biosensor. Finally, due to structural homology, this technique could probably apply to Glucose/Galactose Binding Protein (GBP) too. The engineered GBP (Marvin et al., 1998) has the potential of forming the basis for the development of a new class of fluorescent glucose sensors with potential applications in the food industry or clinical chemistry.

CHAPTER 7

NITRIC OXIDE BIOSENSORS BASED ON THE IMMOBILISATION OF HEMOGLOBIN ON NANOPOROUS TiO₂ FILMS

7.1 Introduction

7.1.1 Nitric oxide

Nitric oxide is a toxic gas and a common air pollutant. It is an endogenously synthesised free radical which plays numerous biological roles in the body, from regulating blood pressure and muscle relaxation (Moncada et al., 1988), preventing platelet aggregation (Radomski et al., 1990), acting as a neurotransmitter (Feldman et al., 1993) and killing tumor cells, intracellular parasites and other pathogens (Hibbs et al., 1987). Therefore, because of its environmental importance and increasing awareness of its biological relevance, there is considerable interest for developing appropriate (fast and selective) sensors for NO. In particular, the interest in developing small sensing NO devices for biomedical use is growing rapidly.

7.1.2 Nitric oxide biosensors

As I mentioned in previous chapters, enzyme-based biosensors are highly selective devices, which rely on the specific binding of the target analyte (substrate) to the active site regions of the enzyme. Optical or amperometric signals resulting from this biorecognition process have led to development of many biosensors. The response of these devices is often affected by the presence of inhibitors which combine with the free enzyme in a manner that prevents substrate binding, having great influence on the velocity of the biocatalytic reaction under study.

A number of studies have focused on the immobilisation of biological recognition molecules, such as proteins, on solid surfaces and electrodes for the development of biosensors for the detection of aqueous NO *in vitro*. Some of them have been used with a different degree of success also *in vivo*.

Richardson et al., 1995 prepared a Nitric Oxide biosensor where Hb or Mb were encapsulated in a sol-gel and absorbance based spectral shifts were used to monitor the binding of NO. The disadvantages are: (a) the requirement for the use of dithionite in order to reduce the encapsulated proteins, because the sol-gels are not electrically conducting to allow electrochemical reduction of the encapsulated proteins and (b) this biosensor's reaction took up to 2 hours to reverse, making dynamic measurements impossible. Other methods for the development of nitric oxide sensors involve a fiber-optic chemiluminescent sensor (Zhou et al., 1996), a peroxidase amperometric biosensor (Casero et al., 2000), an electrochemical sensor based on denatured Cyt-c on gold electrodes (Haruyama et al., 1998), a fluorescence based biosensor incorporating Cyt-c' (Barker et al., 1999a), a fiber-optic biosensor based on Cyt-c' (Barker et al., 1998) and a fiber-optic biosensor based on Guanylate cyclase (Barker et al., 1999b). Despite the great interest in the determination of NO production, all of these methods used for its detection show one or more of the following disadvantages: long response times, irreversibility, high detection limits, short term stability, lack of specificity or selectivity only for NO, reliance on a single fluorescence intensity, or are indirect and rely on measurements of secondary species, such as breakdown products of NO metabolism (nitrite and nitrate).

7.1.3 Optical NO Biosensor Based on Hb/TiO₂ films

The interaction of nitric oxide with biological systems, such as heme proteins, provides a key to the construction of new biosensors, as nature has mastered the creation of highly specific and selective molecules. The binding of NO on heme proteins with Fe centres such as hemoglobin is well established. Hb contains 4 polypeptide chains and four heme groups, which exhibits distinct spectral changes upon NO binding. NO binds to both the ferrous and the ferric derivatives of Hb (Keilin et al., 1937), yielding, in the first case a very stable compound. Nitrosyl Hb can be prepared by mixing the deoxy Fe(II)Hb protein with a solution of the ligand in water. The reaction needs to be performed anaerobically, since NO is readily oxidised by O₂. Deoxy Fe(II)Hb reacts with NO with a stoichiometry of one molecule of NO per heme. Owing to the high affinity of the protein for this ligand (about 1000 times higher than for CO), HbNO is a very stable derivative in the absence of O₂.

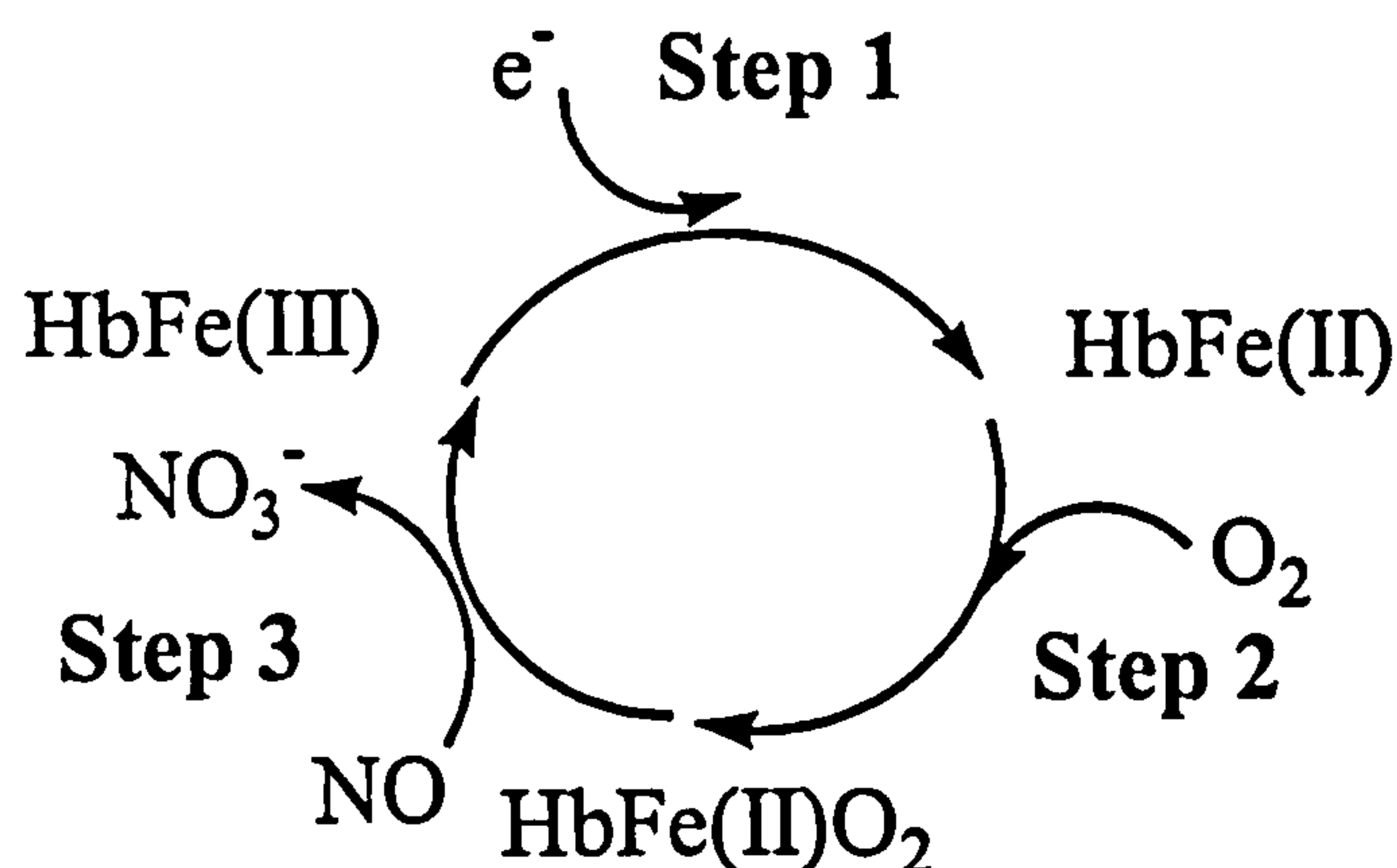
Hb is believed to have a very high affinity for NO compared with other iron-containing proteins and a much higher binding affinity for NO than CO or O₂ (NO > CO > O₂, 3000000 : 2000 : 5.5 mol⁻¹ l) (Antonini et al., 1971). Since the binding of NO with Hb is well established and since immobilised Hb retains its affinity for its ligands (O₂ and CO, see chapter 5), it is possible to create a solid state material which can be used as sensing element for NO. Therefore, an anaerobic optical biosensor, based on the binding of NO on the immobilised deoxy Fe(II)Hb will be presented in this chapter.

7.1.4 Electrochemical NO Biosensor based on Hb/TiO₂ Films

Electrochemical techniques are particularly well suited for the development of analytical methods for the determination of NO because of their sensitivity and the fact that the use of microelectrodes can allow for in vivo applications. The establishment of efficient electron transfer between redox proteins and electrode transducers is crucial for the design of novel amperometric enzyme electrodes.

The oxidative interaction of nitric oxide (NO) with oxyhemoglobin, oxy Fe(II)Hb, to produce nitrate and met Fe(III)Hb is the major pathway for NO elimination from the body (major route of NO catabolism) as well as a reliable method for assaying NO. Scheme 7.1 shows this catalytic cycle of Hb, which has been employed previously with Hb in solution as a colourimetric assay for NO (Balciooglu et al., 1998). This method is limited by the fact that the protein is in solution, making it difficult to extract for reuse and necessitating the use of chemical reagents to carry out redox reactions. Therefore, immobilisation of the protein on the TiO₂ films not only means that it would be easily recoverable from the test solution but also that its oxidation state could be modulated electrochemically.

According to nitric oxide cycle of hemoglobin (see Scheme 1), oxygen binds on the deoxy Fe(II)Hb (step 2) and then oxy Fe(II)Hb reacts with a molecule of nitric oxide to give a nitrite ion and the met Fe(III)Hb (step 3) which is capable of undergoing reduction back to deoxy Fe(II)Hb (step1) and taking part in further cycles (Gow et al., 1999). This presents the opportunity to use films of Hb/TiO₂ to make a sensor of nitric oxide and since Step 1 of scheme 1 is electrochemical it might allow to measure the rate of the reaction, and thus the NO concentration, electrochemically.



Scheme 7.1: Nitric oxide cycle of hemoglobin.

In this thesis, it has already been shown (see chapter 6) that proteins can be adsorbed on nanoporous TiO_2 films and used as optical sensors. Other groups have shown that immobilisation of biomolecules on TiO_2 can be used to probe protein function (Cuendet et al., 1986) and also for catalysis (Mbindyo et al., 1998) and (Mayor et al., 1996). However, the application of nanoporous TiO_2 films on which a protein is directly adsorbed, to amperometric sensing is novel and an important extension of the technology. Therefore Hb/TiO_2 films were used to develop an aerobic, electrochemical biosensor capable of measuring in vitro concentrations of NO in the range 1-10 μM .

7.2 Experimental Procedures

Immobilisation and electrochemical reduction of Hb on the nanoporous TiO_2 films was achieved as described in chapters 2 and 4. The Nitric oxide gas cylinder was obtained from Argo gases. A 1 mM aqueous stock solution of saturated Nitric oxide was prepared anaerobically by bubbling Nitric oxide gas (1 atmosphere) through 2 ml of 10 mM NaH_2PO_4 buffer solution of pH 7 for 1 hour (Wink et al., 1993). Nitric oxide binding on the immobilised Hb was monitored optically by collecting absorption spectra before and after the addition of the saturated solution of Nitric oxide as for carbon monoxide in chapter 6. For the electrochemical NO biosensor, a chronoamperometric technique was used monitoring the current produced by reduction of metHb formed by reaction of oxy (FeII) Hb with nitric oxide.

7.3 Results and Discussion

7.3.1 Optical NO Biosensor

The ability of immobilised Hb on nanoporous TiO_2 films for the sensing of NO in aqueous solution by optical spectroscopy was examined. The heme group of Hb binds NO by electron pair donation. Hb needs to be reduced to its deoxy Fe(II) state in order to show high binding affinity for NO. Figure 7.1 shows that the immobilised Met Fe(III)Hb was initially reduced to its Fe(II), deoxy, state by the application of -0.7 V to the TiO_2 film. Subsequently the addition of a saturating amount of aqueous NO resulted in the characteristic heme absorption changes associate with NO binding.

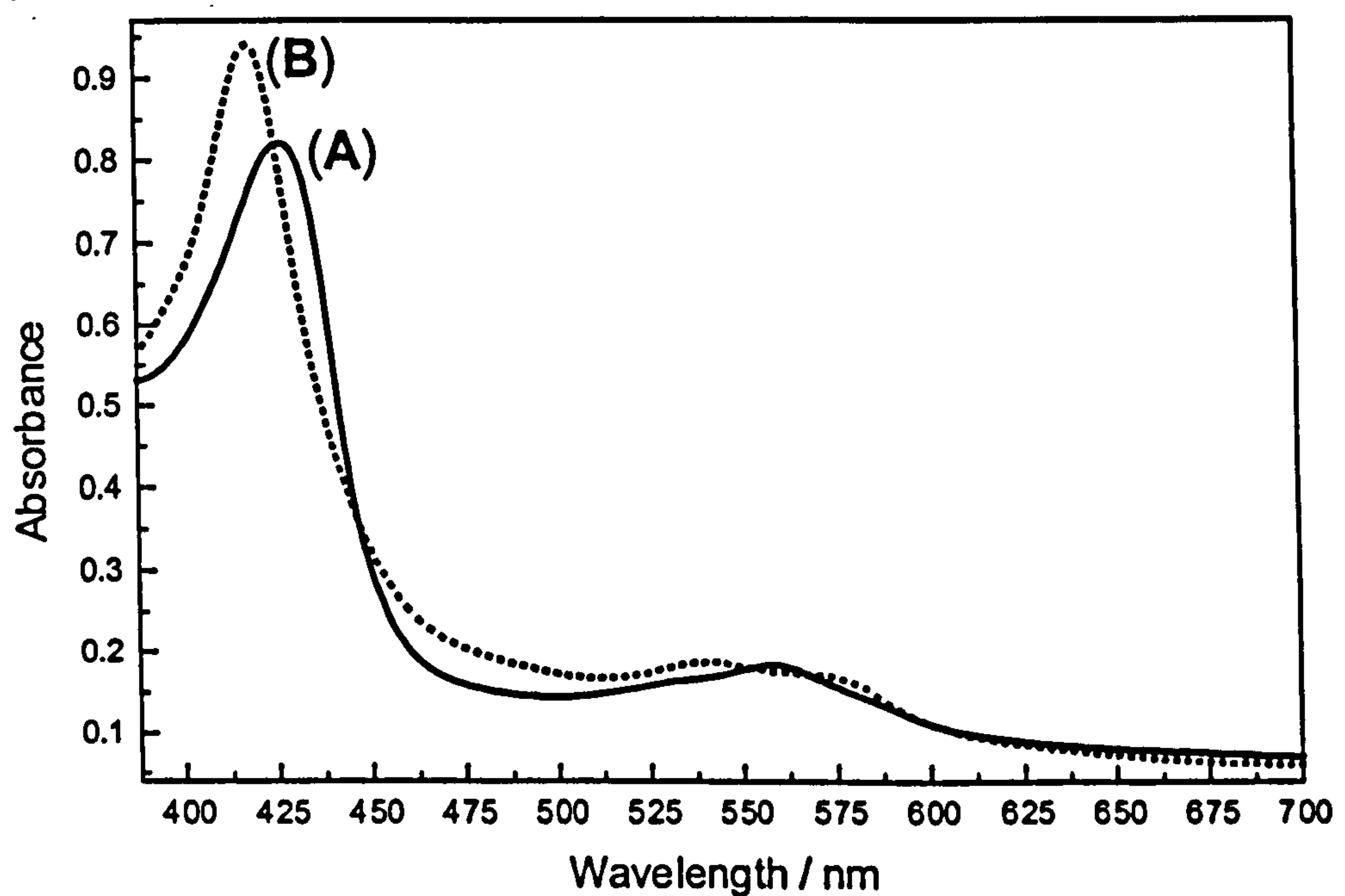


Figure 7.1: Absorption spectra of (A) deoxy Fe(II) Hb and (B) nitrosyl Fe(II) Hb immobilised on a nanoporous TiO_2 film.

Binding of NO to the deoxy *Fe(II)* Hb/TiO₂ film results in a shift in the heme soret absorption maximum from 428 to 418 nm, accompanied by the appearance of α and β absorption bands at 538 and 568 nm (figure 2), in good agreement with solution phase studies of NO binding. These absorption changes could be titrated by the progressive addition of small volumes of 2 mM NO solution to the photoelectrochemical cell, as illustrated in figure 7.2.

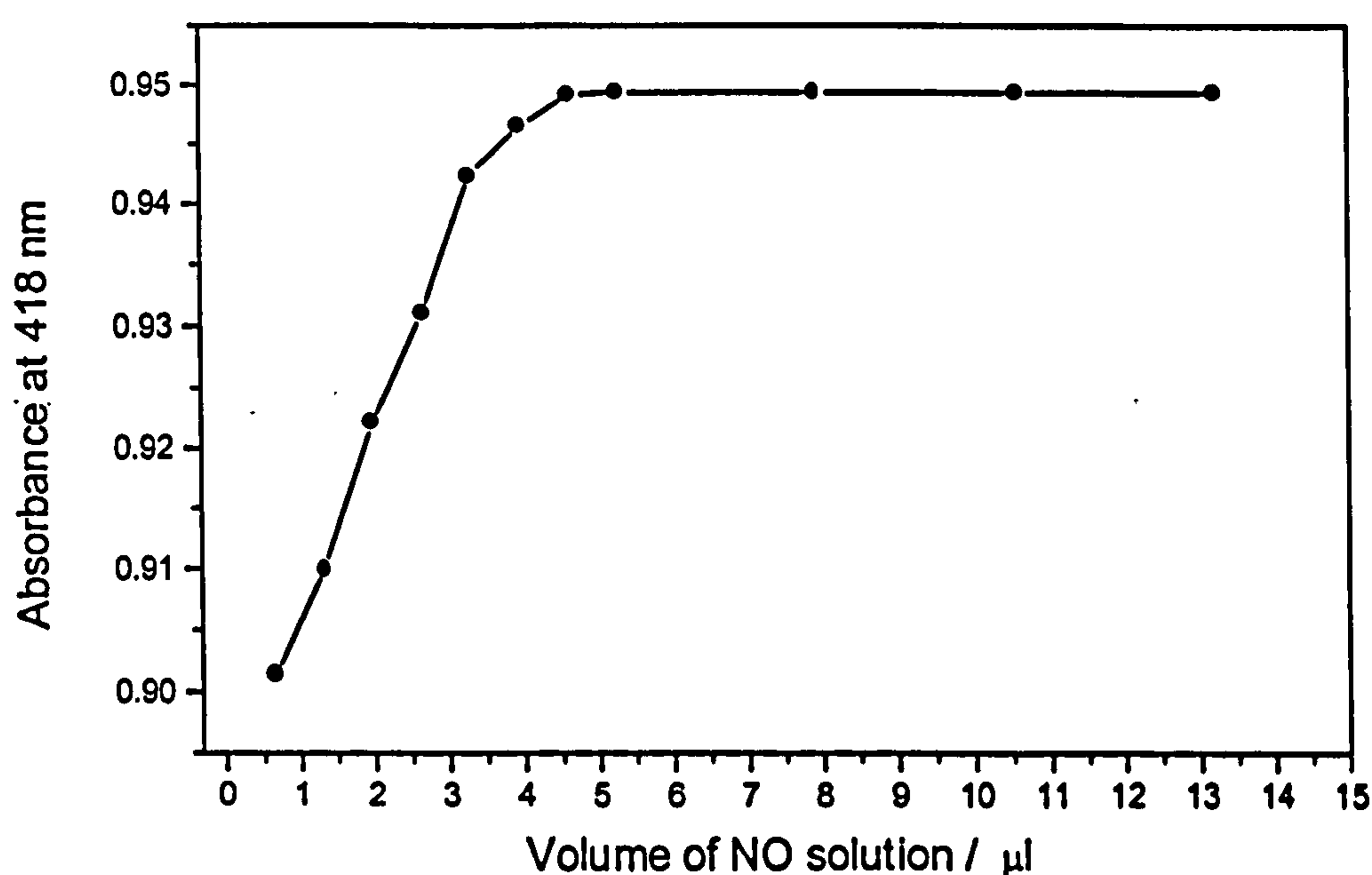


Figure 7.2: Nitric oxide binding curve of Hb immobilised on a TiO₂ film. Deoxy *Fe(II)* Hb titrated against additions of saturated NO solution. The increasing absorption intensity was monitored at 420 nm.

A linear increase in absorbance at 418 nm is observed as a function of NO added to the cell, saturating after the addition of ~ 14 nmoles NO to the 3.5 ml buffer solution in the cell (corresponding to 4 μM olar). The detection sensitivity was approximately 4 nmoles NO. After each addition of NO solution, the absorbance change was complete

within 2 minutes. After the end of the titration the NO was unbound from the immobilised Hb by the application of a positive potential (+0.4 V) resulting in the oxidation of the nitrosyl Fe(II) Hb to met Fe(III) Hb. Following NO desorption, the Hb can be rereduced by the application of -0.7 V, and reused for the determination of NO concentrations. The oxidation process could be further accelerated by bubbling the solution with oxygen whilst applying +0.4 V. However, each complete cycle resulted in 5-6% protein desorption, limiting the repeated use of a single film to 3-4 separate binding titrations.

7.3.2 Electrochemical NO biosensor

By utilising the ability of deoxy Fe(II) Hb to bind oxygen and thereafter react with NO, it is possible to use it as an aerobic sensor for NO. In order to verify this, it is necessary to establish that all steps illustrated in Scheme 1 can be performed using Hb immobilised on nanoporous TiO₂ films.

Oxy Fe(II) Hb was generated in a stepwise procedure illustrated in steps 1 and 2 of scheme 1. First a potential of - 0.7 V vs. Ag/AgCl was applied under Argon to reduce the Fe(III) of Met Hb to the Fe(II) of deoxyHb (Scheme 1, step 1). When the conversion was confirmed spectroscopically to be complete, the potential was brought back to 0 V and oxygen was bubbled through the solution in the electrochemical cell in order to form oxy Hb, the Fe(II)O₂ species (Scheme 1, step 2). The oxy Fe(II) Hb is stable at this potential since the TiO₂ film is significantly insulating and there is no detectable reoxidation of deoxy Fe(II)Hb to met Fe(III)Hb. Complete conversion to oxy Fe(II) Hb

occurs within 2 minutes. Figure 6.1 in chapter 6 shows the typical spectroscopic data for steps 1 and 2 of Scheme 1, showing that in both cases the reaction proceeded to completion.

Further spectroscopic studies confirmed the viability of step 3 of scheme 1 as illustrated by Figure 7.3. This figure shows spectroscopically the reoxidation of oxy Fe(II)Hb to met Fe(III)Hb as a result of its reaction with successive 5 μ l aliquots of a 2 mM NO solution. This re-oxidation is characterised spectroscopically by the loss of absorbances at 540 nm and 576 nm and the shift of the Soret band from 415 nm towards 406 nm, which is the characteristic Soret band of Met Fe(III) Hb (Blyth et al., 1995). It is concluded that Hb/TiO₂ films are able to undergo all the steps of the NO cycle of Hb shown in scheme 1.

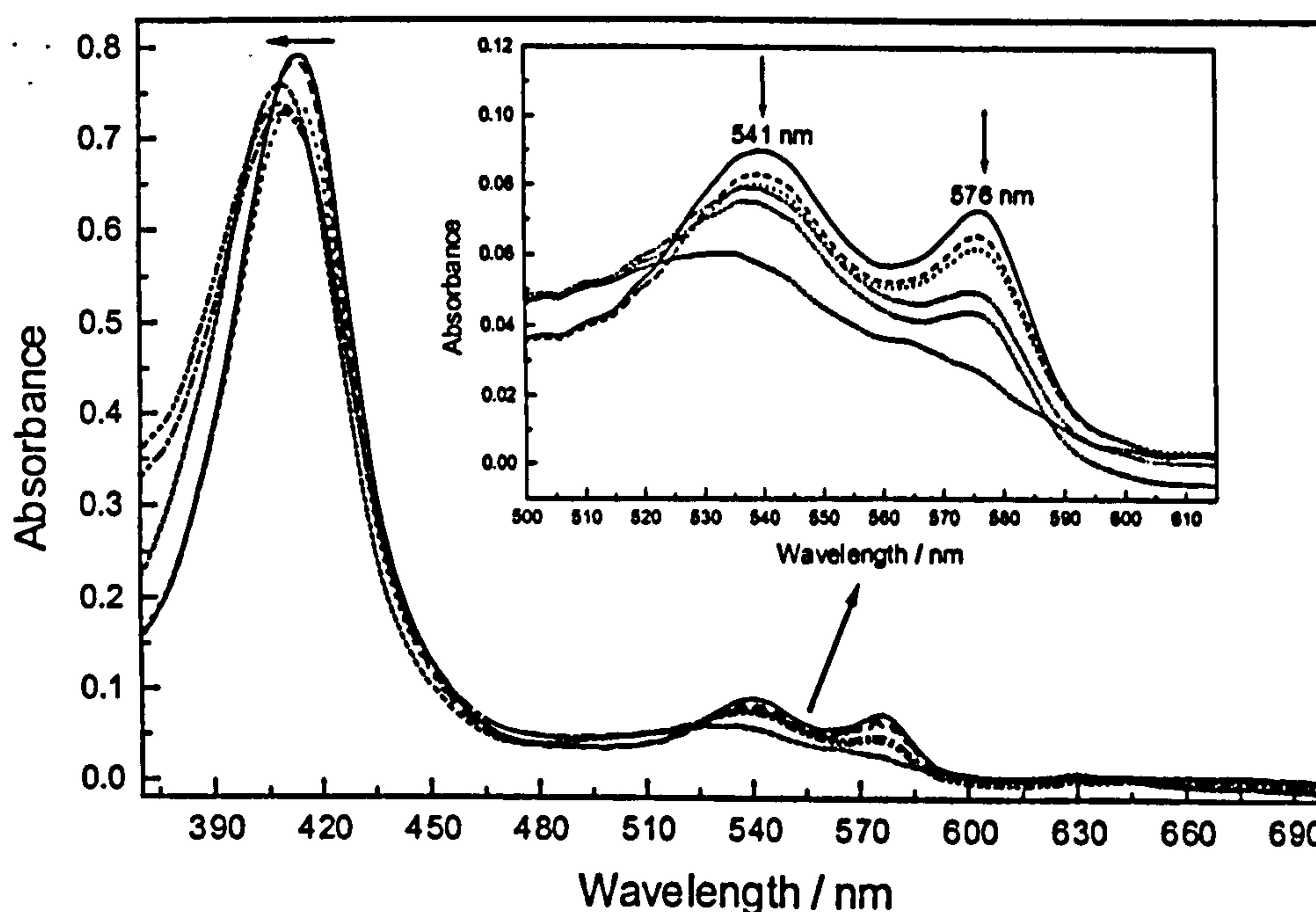


Figure 7.3: Oxidation of oxyHb immobilised on a nanoporous TiO₂ film to metHb by successive additions of nitric oxide. Oxy Fe(II)Hb was generated by electrochemical reduction at -0.7 V, followed by bubbling with oxygen at 0 V. The inset shows the absorbance decrease at 540 and 576 nm on stepwise additions (5 μ l) of NO.

Spectroscopic measurements such as those shown in figure 7.3 correspond to optical sensing of NO concentration and an optical aerobic biosensor can be developed.

By using the same cycle (scheme 1), it would be possible to sense NO electrochemically by monitoring the current flow induced by step 1 of the cycle. If a potential is applied, such that met Fe(III)Hb is reduced *in situ*, the catalytic cycle could be carried out continuously converting NO to nitrate and also monitoring the current caused by reduction. It should theoretically be possible to measure the concentration of NO from the magnitude of the current caused by the reduction of met Fe(III)Hb to deoxy Fe(II)Hb (Scheme 1, step 1).

In order to test this theory, the working electrode of oxy Fe(II) Hb adsorbed on a TiO₂ film was held at -0.1 V vs. Ag/AgCl in a stirred cell using a buffer of 25 mM sodium phosphate at pH 7. To this cell aliquots of a 2 mM NO solution were added in order to make it up to a given concentration. Injections were performed in duplicate and the results are shown in figure 7.4.

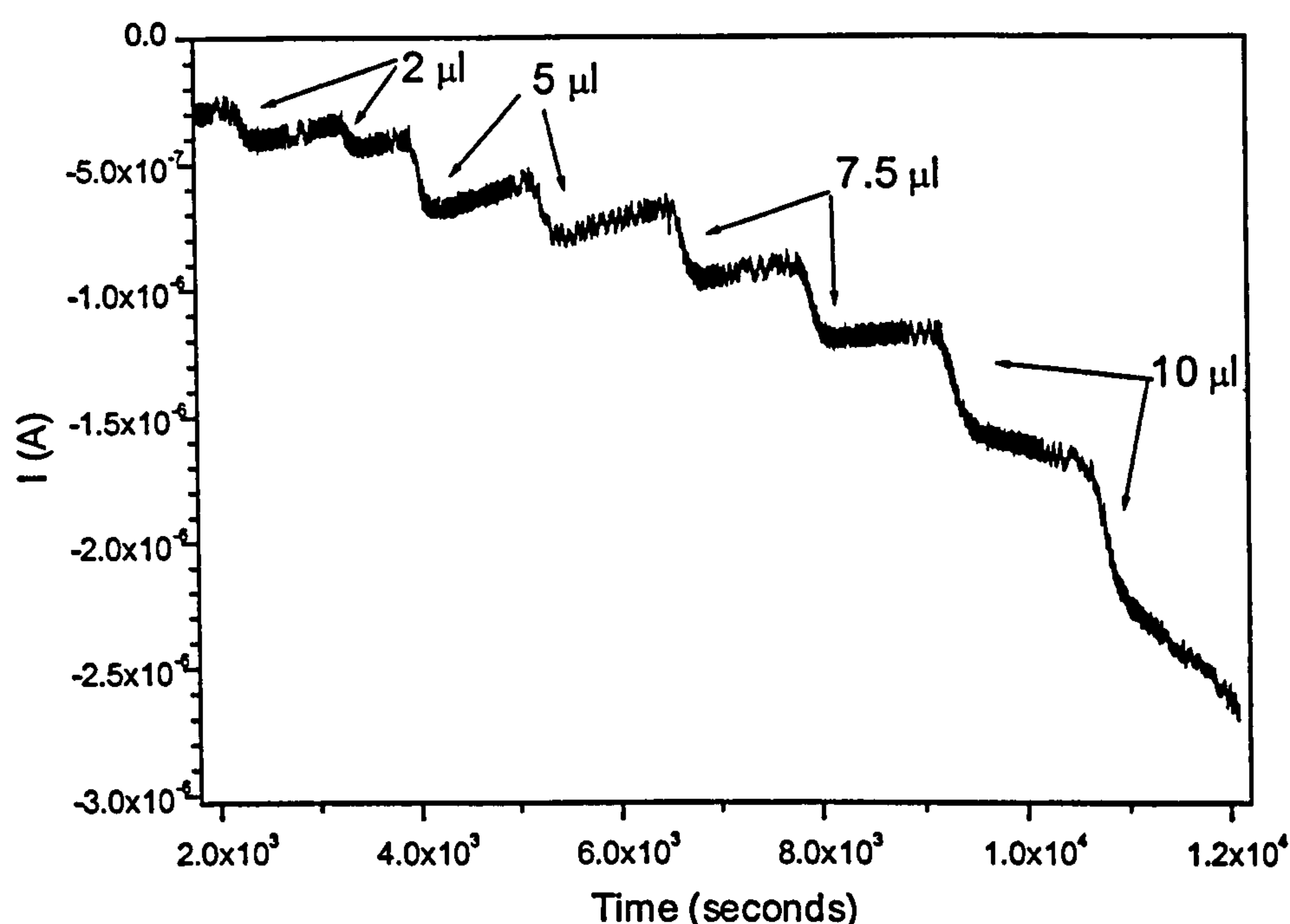


Figure 7.4: Chronoamperometric response of an oxyhaemoglobin/TiO₂ electrode to additions of nitric oxide (2 μl gives a concentration increase of 1 μM in the overall cell concentration).

This representative amperogram monitors the current produced by reduction of met Fe(III) Hb formed by reaction of oxy Fe(II) Hb with NO. The current gets increasingly negative as larger aliquots of NO are added to the stirred cell, the smallest observable current occurring on the addition of 2 μl. In this case an addition of 2 μl leads to an increase of 1 μM in the total NO concentration. The concentration of nitric oxide is calculated on the basis of an addition of typically between 1 and 30 μl of nitric oxide (2 mM) to a cell containing 3.5mls of stirred buffer (sodium phosphate, 10 mM, pH 7).

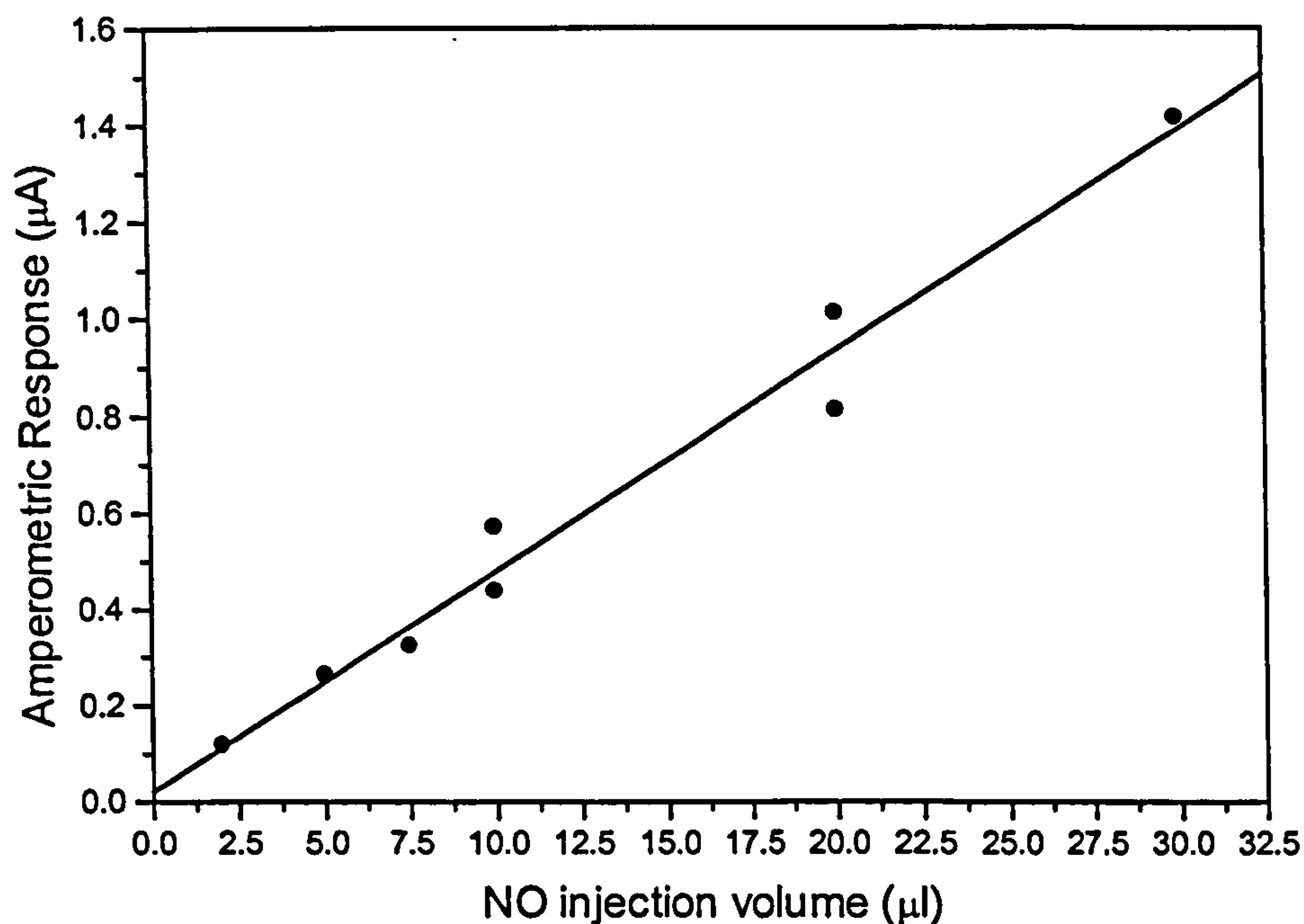


Figure 7.5: Calibration curve of NO aliquot size *versus* amperometric response at -0.1 V vs Ag/AgCl.

According to figure 7.5, the calibration curve shows good linearity for injections of 2-30 μl , a range of 1-15 μM . Above an overall cell concentration of 30 μM the response is no longer reproducible. Although this is a serious limitation to the utility of the device, it is envisaged that moving to a flow-cell based design will avoid the build up of products or unreacted analytes. It is also noted that prolonged usage of the Hb/TiO₂ film resulted in some protein desorption with up to 10% of the immobilised Hb being lost for each complete catalytic cycle. Therefore the improvement of binding stability would be required for such flow cell applications.

In general the advantages of this biosensor are that it works under aerobic conditions, shows a high specificity for NO, its reactions are reversible, the response time is fast (couple of seconds) and the same film can be used for many measurements.

Its disadvantages are the limited conductivity of the nanoporous TiO_2 film and the built-up of products in the closed electrochemical cell.

7.4 Conclusions

The optical anaerobic NO biosensor described in this chapter is unlikely to be applicable to the development of a technological sensor due to its low sensitivity and limited stability. However, like the optical CO biosensor described in chapter 6, it shows the viability of the approach.

Also in this chapter the ability of the immobilised oxyHb to quantify NO in aqueous solutions under aerobic conditions optically or electrochemically has been demonstrated. Although, this work demonstrates both a novel method for detecting NO and a novel use of the nanoporous TiO_2 films, the sensitivity of the biosensor is not yet optimal. The use of different types of nanoporous metal oxide films, with different conductivity characteristics may produce a device with improved sensing characteristics and that the use of flow cell technology may circumvent problems with build-up of products and unreacted starting material. Although this range is outside that required for sensing nitric oxide in the body, *i.e.* sensitivity in the nanomolar range, it demonstrates a clear principle. TiO_2 films can be used to immobilise proteins with clear retention of biological properties and can further be used for electrochemical sensing. Further refinements to this system might improve the sensitivity e.g. moving to a flow-type system may improve signal to noise and expedite removal of reaction products.

Finally, the use of alternative immobilisation strategies would make this technology applicable to a wider range of proteins, which will lead to the development

of many biosensors. The lack of specificity could make the biosensor of limited utility for in vivo systems since they require highly stringent conditions for assurances of selectivity and specificity.

CHAPTER 8

CONCLUDING REMARKS

In this thesis the use of nanoporous TiO_2 films as solid substrates for protein immobilisation has been investigated. Specifically, the protein binding, the electrochemical activity of the adsorbed proteins, the electron transfer kinetics between the proteins and the TiO_2 electrodes and the development of optical and electrochemical biosensors were the main focus of my research. This chapter presents a summary of the most important results already discussed in detail in the previous chapters.

Protein Binding: It is found that protein immobilisation on such films may be readily achieved from aqueous solutions at 4 °C with a high binding stability and no detectable protein denaturation. The binding obeys the Langmuir isotherm and the time required for saturation of binding is roughly dependent on diffusion of the proteins through the film. Among the factors having an important role in controlling the protein binding I have identified the pH, the protein charge, the solution ionic strength, the film thickness and the length of immobilisation time.

Electrochemical Activity: It is demonstrated that the redox state of proteins such as immobilised cytochrome-c (Cyt-c) and haemoglobin (Hb) may be modulated by the application of an electrical bias potential to the TiO_2 film, without the addition of electron transfer mediators. The electrochemical properties of these films were characterised both by cyclic voltammetry and UV/Vis spectroelectrochemistry and seen that both proteins studied retain their electrochemical activity.

Electron Transfer Kinetics: Protein/electrode electron transfer may be initiated by UV bandgap excitation of the TiO_2 electrode. Both photooxidation and photoreduction of the immobilised proteins can be achieved. By employing pulsed UV laser excitation, the

interfacial electron transfer kinetics can be monitored by transient optical spectroscopy, providing a novel probe of protein/electrode electron transfer kinetics.

Development of Biosensors: Optical and electrochemical biosensors have been developed based on protein immobilisation on the TiO_2 films. The fluorescence yield of immobilised fluorophore labeled MBP may be used to monitor specifically maltose and β -cyclodextrin concentrations optically. The potential use of immobilised Hb for the optical detection of dissolved CO and NO in aqueous solutions was also demonstrated. Finally, the electrical conductivity of the films in combination with the catalytic properties of the immobilised Hb have been used for the development of an amperometric NO biosensor.

I hope that the studies presented here can be extended in the future to understand more intraprotein electron transfer dynamics, to develop new generation of biosensors and to be used for photocatalytic applications.

List of Publications

Protein adsorption on nanocrystalline TiO₂ films: an immobilisation strategy for Bioanalytical Devices. Topoglidis, E., Cass, A. E. G., Gilardi, G., Sadeghi, S., Beaumont, N. and Durrant, J. R. *ANALYTICAL CHEMISTRY*, 1998, 70, 5111-5113

Spectroelectrochemistry and photochemistry of haem proteins immobilised on nanoporous titanium dioxide films. E. Topoglidis and J. R. Durrant in *New Directions in Electroanalytical Chemistry II*, J. Leddy, M. D. Porter, and P. Vanysek Eds, Electrochemical Society, Seattle, Washington, Spring 1999, p.152-156

Protein adsorption on nanoporous TiO₂ films: a novel approach to studying photoinduced protein/electrode transfer reactions. Topoglidis E., Lutz T., Willis R. L., Barnett C. J., Cass A. E. G. and Durrant J. R. *FARADAY DISCUSSIONS*, 2000, 116, 35-46.

Factors which affect protein adsorption on nanostructured titania films. A novel spectroelectrochemical application to sensing. Topoglidis. E., Campbell C., Cass A. E. G. and Durrant J. R. *LANGMUIR*, 2001, submitted.

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