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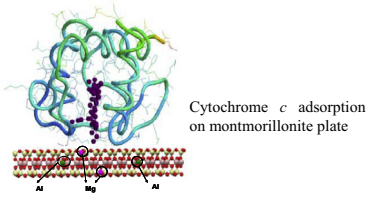
Regular Article

Adsorption of cytochrome *c* on montmorillonite nanoplates: Protein concentration dependence

Svetlana H. Hristova [a,b], Alexandar M. Zhivkov [a,*]

[a] Rostislaw Kaishew Institute of Physical Chemistry, Bulgarian Academy of Sciences (BAS), Acad. G. Bonchev Str., bl. 11, Sofia 1113, Bulgaria
[b] National Specialized Hospital for Active Treatment of Haematological Diseases, Plovdivsko pole Str. 6, Sofia 1756, Bulgaria

GRAPHICAL ABSTRACT



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ABSTRACT

Cytochrome *c* [cyt*C*] is a mitochondrial hemoprotein functioning as electron carrier in the respiratory chain of the biological cells. Being adsorbed on colloid particles cyt*C* can be introduced in the cells by phagocytoses. In the present work we study the adsorption of cyt*C* on montmorillonite (MM) particles combining the electro-optic and electrophoretic techniques. MM particles were chosen as nanoplates having negative pH-independent charge and high ratio surface/mass. The measurements were done at pH 6.5 where cyt*C* globule is positively charged. The main employed method is the electric light scattering based on orientation of colloid particles in sinusoidal electric field. Interfacial electric polarizability was obtained from the degree of orientation at steady-state and the particle size – from the relaxation time after the field switching off. Microelectrophoresis was used to monitor the alteration of the surface charge at protein adsorption. The cyt*C*-concentration dependence of the polarizability and the mobility shows out that the total (net) charge of cyt*C*-MM complex turns its sign from negative to positive, the isoelectric point appears at 5:3 mg/mg (0.135 mol/kg) cyt*C*/MM and saturated protein adsorption is reached at additional twofold increasing of cyt*C*/MM ratio. The suspension is stable at low and high protein concentrations, at intermediate ones aggregation arises.

1. Introduction

The understanding of the adsorption mechanism of globular proteins on solid surface has important significance for wide range of fields: biotechnology (immobilisation of enzymes) [1–3], pharmacy and cosmetics [4], medicine (nano-diagnostics) [5], material science (biocompatible compositions) [6–8], electronics (biosensors) [9,10], geochemistry [11], etc. The structure and the electric properties of the interface have key role for adsorption of biomacromolecules. The specific properties of the clay minerals are the anisotropic layered structure, the polarity surface and the intralayer electric charge [6]. The clay colloid particles have huge ratio area/mass [12] and pH-independent charge (determined by atom substitution in the crystal lattice) which density is relatively high [13]. The negative charge is due to substitutions of SiO_2 with AlO_2^- in the tetrahedral sheets and of AlO(OH) with $MgO(OH)^-$ in the octahedral ones. The excessive charge is distributed over the metal atom and the surrounding electronegative O-atoms so that

* Corresponding author.
E-mail address: zhivkov_ipc@doctor.bg (A.M. Zhivkov).

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the metal atom then carries a reduced positive charge [6]. In the spaces between the layers their negative charge is compensated by cations such as K^+ and Na^+ disposed closely the substituted metal atoms [14]. When the charge constancy is required the clay minerals [15] have advantage over the metal-oxide colloids which surface is amphoteric: the surface charge depends on pH in wide range from positive to negative [16] but with lower charge density [17]. Due to the high surface area and permanent electric charge the clay materials are very good adsorbents and are used in many industries. A special attention attracts their application in pharmacy and medicine as a drug delivery system [18–23].

Often used clay mineral is montmorillonite (MM) due to the capability of its plate-like particles to be split up to nanoplates and by that extremely increase the ratio area/mass [24]. The MM nanoplate is built by one central alumina (Al^{3+} in octahedral coordination with O^{2-}) and two lateral silica (Si^{4+} in tetrahedral coordination with O^{2-}) atom sheets. The negative charge is due to a partial substitution of Al^{3+} in octahedral sites by Mg^{2+} and Fe^{2+} and to a lesser degree, by partial substitution of Si^{4+} in tetrahedral sites by Al^{3+}. The MM-plates immersed in aqueous medium carry two kinds of electric charges: a negative charge on the surface resulted from isomorphic substitutions and pH-dependent charge on the edges resulting from H^+ or OH^- adsorption/desorption [25]. The increment of edge charge is small owing to high ratio between the micrometric diameter and nanometric thickness of the plates. That is why the MM particles are described usually as bearing pH-independent negative charge [26].

The edge-charge can play significant role only at pH < 6–7 when it obtain positive sign because that is precondition for face-to-edge aggregation when the positively charged edge contact with negative surface of one or more neighbour particles [27–29]. Such type of aggregation leads to formation of so-called card-house structures and can significantly alter the rheological properties of the MM suspension but it does not reduce its adsorption capability due to the unaltered ratio area/mass. Moreover, the face-to-edge aggregation appears only in concentrated MM suspension at enough high ionic strength (>0.1 mol/L) due to the predominance of the local negative charge over the positive one near to the plate edges [28]. Face-to-face aggregation reduces the adsorption capability but it is possible only at much higher ionic strength (>1 mol/L) required to diminish the strong electrostatic repulsion between the surfaces of two neighbour particles [28].

The charge density of MM colloid particles depends on the kind and number of the isomorphic replacement in the crystal lattice [30] and especially on the origin of the mineral; a typical values lay in the range $(0.8–2) \times 10^{-2}$ C/m^2 [31,32]. This charge density determinates electrophoretic mobility which is usually 2–3 (μm/s)/(V/cm) [33,34] and corresponds to zeta-potential from $\zeta = -30$ to -70 mV at ionic strength 10^{-4} mol/L, falling to $\zeta = -28–40$ mV at 10^{-2} mol/L [35,36].

Selecting the colloid particles for protein adsorption we have compared MM with kaolinite. The both particles have plate-like form, lamina structure, structurally determined negative charge and pH-dependent once on the edges. The difference is that kaolinite particles cannot split up to mono-plates and their surface charge density is lower; that facilitates card-hose and face-to-face aggregation at relatively low ionic strength. As a result the kaolinite suspension is not stable at pH < 8 [34,37,38] (when the edge-charge is positive) while the MM suspension is stable in the hole pH-range at ionic strength under 0.1 mol/L due to the higher surface density of the pH-independent negative charge. The difference between the electric properties of the two clay minerals predetermines their application for different goals: the MM – when suspension stability is required in wide pH and ionic ranges, while the kaolinite is often used for studying the flocculation caused by different agents, in particular by linear charged polymers [39,40]. For adsorption of positively charged proteins the MM is more appropriate due to the higher ratio surface/mass and the higher negative charge density, both determining higher number of adsorbed macromolecules on a unit colloid mass.

The electric charge of proteins is determined by proton desorption/adsorption from/on the carboxylic (COOH) and amino (NH_2) groups of the amino-acid residues accessible to the surrounding aqueous medium. The dissociation constant (correspondingly, pK_a defined as pH at which the ratios COO^-/COOH and NH_3^+/NH_2 are equal to unity) of these groups is determined by both affinity to H^+ and the local electrostatic field. That means that pK_a of every dissociable group is specific and cannot be known without special calculations. The net charge of the protein globule at given pH is determined by the difference of positively and negatively chargeable groups and their specific pK_a, correspondingly, the isoelectric point pH_i is also specific for a given protein.

The native three-dimensional conformation of the polypeptide chain is determined by hydrogen bounds and hydrophobic, vander-Waals and electrostatic forces between amino-acid residues [41]. The protein stability depends on pH of the aqueous medium because the ionization of the dissociable groups alters the electrostatic forces. The protein macromolecules retain their conformational stability in limited pH-range, specific for each of them, and undergo irreversible denaturation at exceeding out of the range; the critical values pH_{ad} and pH_{bd} are defined as pH-points of acid and base denaturation, respectively.

At adsorption on solid surface the proteins can undergo conformation changes in different degree up to full denaturation. The alterations depend both on the specific protein structure and the electric charge of the substrate; denaturation appears as a rule on a metal surface and rarely on dielectric one. The clay minerals, beside the huge ratio area/mass of their colloid particles, have an advantage being sparing substrate for adsorbed protein macromolecules.

The adsorption of many proteins on MM colloid particles was experimentally studied. Part of them as β-lactoglobulin, glucosidase, bovine serum albumin (BSA) are structurally unstable and change their conformation on the surface [1,42,43]. Other proteins are stable and remain their biological activity being adsorbed on the MM particles: phosphatase, urease, catalase and *c*-type cytochromes [44–47]. For the goals of our research we have chosen cytochrome *c*-type because of its conformational stability and important biological function.

Cytochrome *c* [cyt*C*] is a small water soluble globular protein containing 104 amino-acid residues and one *c*-type heme-group (Fe-protoporphyrin) [48]. It is localized in the gap between inner and outer mitochondrial membranes and functions there as electron carrier between membrane-integrated proteins of the respiratory chain. This function cyt*C* performs due to possibility of Fe-atom to undergo oxidation/deoxidation ($Fe^{+2} \leftrightarrow Fe^{+3}$) reactions by accepting or releasing one electron.

The cyt*C* have 30 chargeable amino-acid residues, the isoelectric point appears at pH 10, at pH 6.5 the globule is positively charged having 16 positive charges and 9 negative ones [49]. The cyt*C* undergo acid denaturation under $pH_{ad}2$ and base denaturation above $pH_{bd}12$ [50–53]; no structural changes take place between pH 3 and pH 12 [54,55]. The deference [$pH_{bd}12–pH_{ad}2$] demonstrates pH-range of stability as wide as ten pH-units where the macromolecule does not show noticeable conformational changes and retains its functional activity.

The extremely high structural stability of cyt*C* in solution predicts a conformational stability of the globule being adsorbed on a solid surface. The experimental studies conforms this expectation. The electrochemical measurements have shown out that cyt*C* retains its oxidation/deoxidation capability being immobilized on