Materials Science and Engineering C 33 (2013) 4965–4971

Contents lists available at ScienceDirect

Materials Science and Engineering C

journal homepage: www.elsevier.com/locate/msec

Studies on the chemico-biological characteristics of bilirubin binding with collagen

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ARTICLE INFO

Article history:
Received 3 May 2013
Received in revised form 11 July 2013
Accepted 18 August 2013
Available online 28 August 2013

Keywords:
Collagen
Bilirubin
Spectroscopy
Fluorescence
Surface plasmon resonance

ABSTRACT

The clinical impact of bilirubin on collagen is investigated using various physical, chemical and biological methods. Thermo gravimetric analysis and differential scanning analysis of collagen–bilirubin complex matrices indicate that crosslinking does not alter their thermal behavior of collagen. The polydispersity of collagen–bilirubin complex increases in the reacting medium suggesting that there is an increase in the number of interacting points between them. Based on the zeta potential values, the rate of mobility of interacted complex decreases by inferring the extent of binding compared to the control collagen. Emission intensity begins to increase with increase in concentration of bilirubin which ascribes the conformational changes around the aromatic amino acids in collagen. Binding is indicated by an increase in resonance units and the responses are corrected by subtraction of those obtained for native collagen. Bilirubin showed a higher affinity for collagen at a concentration of about 25 nM/mg. In this study, the association rate has been calculated which depicts the increased affinity of bilirubin to collagen. Affinity for bilirubin to collagen has been found to be 8.89×10^{-3} s^{-1}. The greater part of binding of bilirubin to collagen is found to be electrostatic in nature. The investigation leads to comprehend the affinity of collagen–bilirubin complex during jaundice diseased tissues.

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1. Introduction

Bilirubin, a toxic metabolite of heme has been studied extensively for its binding with Human Serum Albumin (HSA) for many years. This resulted in bilirubin encephalopathy in premature infants [1]. In this condition, higher concentration of bilirubin causes toxicity to cells. Moreover, it partitions from the blood to neuronal tissue causing irreversible damage to brain. Liver is the organ responsible for conversion of bilirubin to a soluble form and its excretion. The unbound bilirubin is found to absorb to the skin [2–4]. This indicated that the extensive yellowing of the body surface in hyperbilirubinemic newborns and the restorations to the normal color of the skin after recovery from jaundice. This suggests that skin takes active part in the homeostasis of bilirubin at least during the diseased condition [5]. Evidence has been adduced earlier to show that skin epithelium and skin strips of the mouse, rat, guinea pig and man possess a mechanism to accumulate and release bilirubin.

Analyses of the interactions of ligands with biomacromolecules include the use of various spectroscopies as an important tool [6–10]. Interaction of bilirubin with collagen is of high pharmaceutical importance. The binding parameters derived from the spectroscopic methods play a major role in pharmacokinetics [11,12].

In the present study, the physical, chemical and biological methods have been used to analyze the bilirubin bound to collagen matrix and they have been intercompared. The differences obtained have been used to draw conclusions about the contribution of various amino acid residues to the structure of the collagen–bilirubin complex. Moreover, in this study the binding characteristics of collagen and immobilized bilirubin are studied for their effective regeneration on the sensor surface, and the apparent association and dissociation rates.

2. Materials and methods

2.1. Materials

Type I collagen and bilirubin are purchased from Sigma Aldrich (St. Louis, MO, USA) and Tokyo Kasei (Tokyo, Japan), respectively. Acetic acid is supplied from Wako Pure Chemicals (Osaka, Japan). Sodium chloride (NaCl) is obtained from Nacalai Tesque (Kyoto, Japan). All chemicals are used without further purification.

2.2. Solubilization and purification of collagen

Tails are excised and frozen at −20 °C from 6-month-old male albino rats (Wistar strain) that are ideal collagen substrate for crosslinking

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http://dx.doi.org/10.1016/j.msec.2013.08.021

studies due to its high purity, available lysine residues and collagen content. On removal from the freezer, the tails are thawed and tendons are teased out. Teased collagen fibers are ished with 0.9% NaCl at 4 °C, to remove the adhering soluble proteins. Rat Tail Tendons (RTTs) are washed extensively in double distilled water at 4 °C and used as collagen fibers. Acid soluble RTT type I collagen solution is also isolated according to the method described by Chandrakasan et al. [13]. The procedure included acetic acid extraction and salting out with NaCl. The purity of collagen preparation is confirmed by SDS–Polyacrylamide gel electrophoresis (PAGE). The collagen concentration in the solution is determined from the hydroxyproline content according to the method of Woessner [14].

2.3. Preparation of bilirubin solution

The recrystallized bilirubin (about 5 mg) is dissolved in 1 mL of 38 mM sodium carbonate solution containing 1 mM EDTA (pH 11.0). This solution is centrifuged at 5000 rpm for 10 min to remove insoluble bilirubin. The pH of the bilirubin solution is adjusted to 8.0 with Tris buffer saline (10 mM Tris, 150 mM sodium chloride, pH 7.6). The concentration of bilirubin is determined spectrophotometrically using molar extinction coefficient of $6.2 \times 10^4\ M^{-1}\ cm-^1$ at 453 nm [15].

2.4. Preparation of reconstituted collagen membrane

Transparent membranes are prepared by adding collagen solution (1 mg/mL) along with different concentrations of bilirubin (0–25 nM) at pH 7.4 on flat polyethylene plates and the resulting films are dried in the laminar hood by blowing air over the surface. The plates are then placed over the nitrogen flow to neutralize acetic acid. Since collagen does not adhere to polyethylene, the dried films could easily be taken off from the plates.

2.5. Thermal properties

2.5.1. Differential scanning calorimetry

Thermal analysis is subjected for assessment of strength properties of collagen membrane treated with different concentrations of bilirubin (0, 5, 10, 15, 20 and 25 nM) by measuring the denaturation temperature (*Td*). The change in *Td* indicates alteration in collagen strength. Thermal transitions of samples within each collagen–bilirubin ratio group are measured using a differential scanning calorimeter (DSC) using TA Instruments DSC-Q2000 Differential Scanning Calorimeter (METTLER TOLEDO Company, Switzerland) over a temperature range of 25 to 150 °C at a heating rate of 10 °C/min. *Td* is recorded at maximum peak height of the denaturation endotherm [16,17].

2.5.2. Thermo gravimetric analysis

Thermogravimetric analysis (TGA) is performed to determine the interaction potency of collagen membrane with bilirubin of different concentrations such as 0, 5, 10, 15, 20 and 25 nM/mg of collagen. It measures the amount and rate of change in the weight of a material as a function of temperature or time in a controlled atmosphere. Measurements are used primarily to determine the composition of materials and to predict their thermal stability at temperatures up to 150 °C. The technique can characterize materials that exhibit weight loss or gain due to decomposition, oxidation or dehydration. The membranes, each with a weight of 10 mg, are studied using a TA Instruments TGA-Q600 Thermo gravimetric analyzer (METTLER TOLEDO Company, Switzerland) and the measurements are recorded from 20 to 150 °C with a heating rate of 10 °C/min in air. Primary weight change of the samples as a function of temperature is recorded using this study [18].

2.6. ATR-FTIR (Attenuated Total Reflectance Fourier Transform Infrared) analysis

FTIR spectra of collagen and the bilirubin interacted collagen matrices (5, 10, 15, 20 and 25) are obtained using ATR-FTIR spectrometer (Jasco Spectrometer, Japan). The horizontal attenuated total reflectance accessory (HATR) is mounted into the sample compartment. The internal reflection crystal (Pike Technologies, Madison, WI, USA), made of diamond, had a 45° angle of incidence to the IR beam. Spectra are acquired at a resolution of 4 cm^{-1} and the measurement range is 4000–650 cm^{-1} (mid-IR region) at room temperature. Automatic signals are collected in 32 scans at a resolution of 4 cm^{-1} and are plotted against a background spectrum recorded from the clean empty cell at 25 °C. All spectra are collected by co-adding 128 scans at a resolution of 2 cm^{-2} and a gain of 1.0 [19]. Twelve replicates of each sample are analyzed and spectra for the replicated runs are averaged. Fourier self deconvolution is conducted on the average spectra for the amide I band, using a resolution enhancement factor of 1.8 and full height band width of 13 cm^{-1}.

2.7. Dynamic light scattering analysis

Dynamic light scattering (DLS) measurements are analyzed using Delsa Nano C particle analyser, Beckman counter with a He–Ne laser (632.8 nm, 35 mW) as light source. Monodisperse collagen solutions (1 mg) obtained through purification are interacted with different concentrations of bilirubin (0, 5, 10, 15, 20 and 25 nM/mg of collagen), studied by dynamic light scattering. The light scattering explains that when electromagnetic radiation impinges upon a collection of molecules, the light is scattered from the collagen–bilirubin complex at all angles. The scattered light can be used to extract information about the physical properties of the scattering sample. The primary concern is with the scattering of light from solutions of collagen and collagen–bilirubin complex. The major apprehension dealt with its polydispersity nature and its size and shape evolved as a function of time. Dynamic light scattering measurements yield a value for the diffusion coefficient, D, of the particles which is directly related to the frictional coefficient, f given by $D = kT/f$, where k and T are Boltzmann's constant and absolute temperature respectively [20].

2.8. Zeta potential measurements

Colloidal particles accumulate charge at their surface, which can be expressed as surface potential. This property is evaluated for the collagen solution as a function of bilirubin concentrations (0, 5, 10, 15, 20 and 25 nM/mg of collagen). The zeta potential of samples is determined with a Zeta Potential Analyzer from Delsa Nano C analyzer, Beckman counter. ξ is automatically calculated from electrophoretic mobility based on the Smoluchowski equation, $v = (\varepsilon E/\eta)\,\xi$, where v is the measured electrophoretic velocity, η is the viscosity, ε is the electrical permittivity of the electrolytic solution and E is the electric field [21]. Measuring the colloidal charge typically involves applying an electrical voltage to the particle and determining the speed of the induced movement. The cell of 5 nm × 2 nm rectangular quartz capillary is used for the study. The temperature of the experiments is 298 ± 0.01 K.

2.9. Microscopic fluorescence detection and spectral analysis–Erecting Fluorescence Microscope (DM2500 Fluo/DIC)

The fluorescence emission spectrum of collagen overlaps significantly with the absorption spectrum of bilirubin. Hence, the net increase in fluorescence of collagen upon serial additions of bilirubin is directly proportional to the fraction of collagen molecules with a bilirubin molecule bound. Here, the fluorescence analysis is achieved using three techniques: (1) spectral analysis which is used to measure the emitted light intensities at intervals of 7 nm across the total spectrum, generating curves via intensity profiles; (2) intensity comparison

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