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A noninvasive method for measuring the oxygen binding-releasing capacity of hemoglobin-loaded polymeric nanoparticles as oxygen carrier

Xiaolan Zhang · Changsheng Liu · Yuan Yuan · Xiaoqian Shan · Yan Sheng · Feng Xu

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Abstract Based on the strong penetration capacity of near infrared lights (NIRs) and different absorption of oxyhemoglobin and deoxyhemoglobin in NIRs region, a novel noninvasive method, with the aid of an airproof-equilibrium apparatus, was developed to determine the oxygen binding-releasing capacity, including oxygen dissociation curve (ODC) and P_{50}, of the hemoglobin-loaded polymeric nanoparticles (HbP) in this study. The measured ODC of the PLA-PEG HbP was very close to that of the native hemoglobin, and the corresponding P_{50} (26.1 mmHg) was also near to the native precursor protein (27.3 mmHg), indicative of the validity of the method proposed. To further verify the method proposed, the oxygen binding-releasing capacity of the HbPs prepared by PCL, PCL-PEG, PLA were also investigated with human blood as control. These results indicated that the method developed here enabled accurate and noninvasive determination of the oxygen binding-releasing capacity of the biodegradable polymeric oxygen carriers.

1 Introduction

To relieve shortage of donor blood and avoid risks of transmittable diseases associated with the use of allogenic blood in trauma care, great efforts have been made to develop artificial oxygen carriers over the years [1, 2]. Stroma free hemoglobin (Hb), when injected into body, however, was susceptible to dissociate from the tetramer into dimmer structure and rapidly eliminated from the circulation, filtered by the kidney and induced renal toxicity and other adverse effects [3]. To overcome these problems, Hb encapsulated in biodegradable polymers, simulating the structure of RBCs, shows a promising future because Hb can be maintained in a protected, predominantly aqueous environment [4, 5].

The maintenance of the oxygen binding-releasing capacity of Hb during the treatment is a key requirement, and a valid method to evaluate this property is also of importance. The common description of the oxygen binding capacity of human whole blood or Hb-based oxygen carriers is P_{50} and it can be directly obtained from the oxygen dissociation curve (ODC), which reflects the relationship between oxygen partial pressure (P) and the oxygen fractional saturation (Y). Thus determination of P and Y renders the information of ODC as well as P_{50} available. P is easy to be measured by routine oxygen electrodes. As for Y, to date, only two available instruments, the commercial blood-gas analyzer and the pulse oximeter, in clinic practice can be used to detect it. The commercial blood-gas analyzer includes an old and a new model. The old blood-gas analyzer, which can directly detect pO_2, pCO_2 and pH values in the blood by the corresponding electrodes, and the Y is estimated according to the standard oxygen dissociation curves of the whole blood, is mainly applied to human blood [6]. The new one, which can easily quantify the Y by measurement the exposed Hbs through breaking RBCs by ultrasonic fixed, has successfully used in the whole blood, lecithin-encapsulated oxygen carriers and micro-scale particles oxygen carriers, etc. The pulse oximeter, based on the strong penetrating ability of near infrared lights (NIRs), can penetrate through tissues such as skins, muscles, bones and blood vessels to measure the Hbs in RBCs [7] and the

X. Zhang (✉) · C. Liu · Y. Yuan · X. Shan · Y. Sheng · F. Xu
Key Laboratory for Ultrafine Materials of Ministry of Education, Engineering Research Center for Biomedical Materials of Ministry of Education, East China University of Science and Technology, Shanghai 200237, People's Republic of China
e-mail: zxlpsh@126.com

C. Liu
e-mail: liucs@ecust.edu.cn

differential optical absorption of oxyhemoglobin (oxyHb) and deoxyhemoglobin (deoxyHb) in NIRs regions [8], can provide continuous invasive detection of Y.

In the previous study, our group has centered our attentions on the fabrication of biodegradable polymeric HbP by modified solvent diffusion-evaporation double emulsion technique. HbP prepared was accompanied with high encapsulation efficiency, controlled particle size and connecting porous structure [9, 10]. After that, we try to investigate the oxygen binding-releasing capacity of this HbP obtained through the above-mentioned commercial instruments. When the new commercial blood-gas analyzer was used, unfortunately, the signal was very weak. The potential explanation for this phenomenon might involve the nano-scale diameter of the particle (about 200 nm) and firm polymer matrix (or shell), like PCL shell, which make it very difficult to destruct the HbPs and to expose the entrapped Hbs by ultrasonic. In addition, the ultrasonic stress might induce the change of the local oxygen pressure and thereby lead to the variation of the oxygen saturation. So this destruction-extraction method is not suitable for the detection of the oxygen-carrying capacity of the HbP. As for the pulse oximeters, although the artificial pulse mimicking human pulse was designed employing some mechanical devices by our laboratory initially (the detailed description not shown), the continuous human pulse, and the continuous change of the relative content of oxyHb and deoxyHb, unfortunately, was difficult to simulate in vitro. Therefore, development of an alternative strategy to detect the Y for the nano-scaled polymersome HbP oxygen carrier is greatly needed.

According to the differential optical absorption of gaseous ligands to Hb (oxyHb) and nongaseous ligands (deoxyHb) in visible light region, Giardina and his co-workers have established a classical spectrophotometric method to determine the Y of cell-free Hb [11]. The main advantages of the technique essentially included the rapid and easy procedure, controlling of the protein state at any time, small quantities of sample and a wide range of protein concentrations. Herein, a method, combining this conventional simple spectroscopy procedure and the strong penetrating ability of the NIRs, with the different optical properties of oxyHb and deoxyHb in the region of NIRs as well, was established to detect the Y, and subsequent the oxygen binding-releasing capacity of nano-sized polymeric oxygen carrier in the present study.

2 Materials and methods

2.1 Materials

Bovine Hb (BHb) in lyophilized form (64.5 kDa) was purchased from Yuanju Biotechnology Company, Shanghai. Poly(ε-caprolactone) (PCL), M_W 45 k, poly (ε-caprolactone-ethylene glycol) (PCL-PEG), M_W 42 k, M_{WPEG}:M_{WPCL} = 1:6, Poly (DL-lactide) (PLA), Mw 48 k, and Poly (DL-lactide-ethylene glycol) (PLA-PEG), Mw 43 k, M_{WPLA}:M_{WPEG} = 7:3 were obtained from Chengdu Institute of Organic Chemistry, Chinese Academy of Science, Chengdu. The composition and molecular weight were characterized according to the specification of manufacturers. Healthy human blood was a gift from shanghai Dahua hospital. All other reagents were of analytical grade.

2.2 Preparation of Hb-loaded nanoparticles (HbP) and characterization

HbP was prepared by the modified five-step solvent diffusion-evaporation double emulsion technique. Briefly, 0.5 ml BHb solution (150 mg/ml) or phosphate buffer (for blank HbP) was emulsified in 5 ml of organic solvent containing 10 mg polymer by ultrasonic (JYD-900, ZhiXin Instrument Co., Ltd., Shanghai) for 15 s. Thereafter, the primary emulsion was poured into aqueous solution followed by two steps of re-emulsification by a high-speed homogenizer (WX500CY, Shanghai Weiyu company, China) for 15 s and 2 min, respectively. The double emulsion was subsequently added to 200 ml aqueous solution to remove the solvents. The nanoparticles were finally recovered by centrifugation (GL-21 M, Shanghai Centrifuge Institute Company, Shanghai) at 25,000 *g*, washed three times with deionized water, and lyophilized using a freeze-drier (FD-3, Beijing Boyikang instrument company, China). The detailed preparation can be found in the reference formulation [9, 10]. The morphology and size of the nanoparticles were observed by transmission electronic morphology (JEM-2010, Japan), size and size distribution were studied by dynamic light scattering analyzer (Zetasizer Nano ZS, Malvern Instruments Ltd).

2.3 The airproof-equilibrium apparatus design

Airproof-equilibrium apparatus (with 50 ml total volume) is made of organic glass and mounted by a vacuum manometer in the upper to control gas pressure introduced, gas inlet/outlet with valves, sample outlet to introduce or withdraw samples, and oxygen meter with a digital readout (HI93732 N dissolved oxygen meter, Hanna Instrument, Italy) at the bottom to detect the partial pressure of oxygen dissolved. The gas inlet is connected with gas (O_2, CO_2 or N_2) tanks to obtain various O_2 partial pressures. The representative description of the apparatus was shown in Fig. 1.

2.4 Assay the oxygen dissociation curve and P_{50}

Oxygen dissociation curve was measured in an airproof-equilibrium apparatus. Cell-Free native BHb or HbP

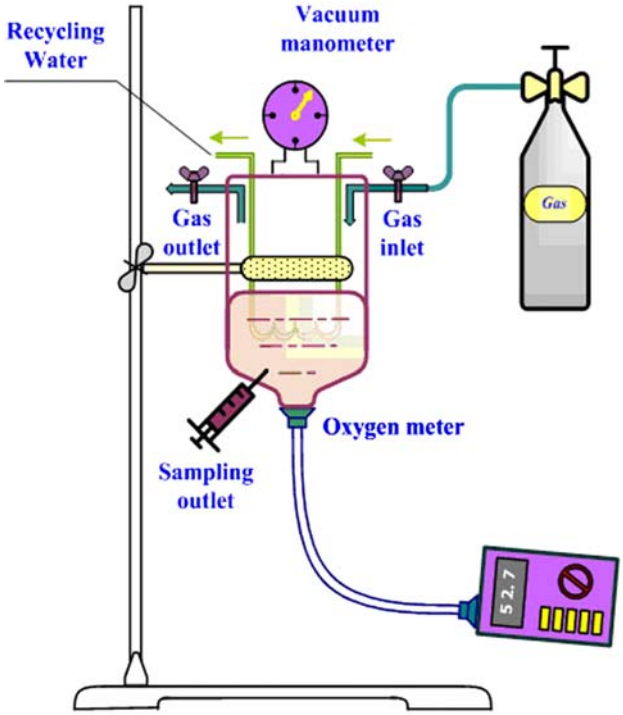

Fig. 1 Schematic representation of airproof-equilibrium apparatus to measure the oxygen binding-releasing capacity of Hb-loaded nanoparticles (HbP)

(50 uM, Hb) were resuspended in phosphate buffer (pH 7.4, 0.1 M Cl^{-1} used as allosteric effector to regulate the oxygen affinity for BHb) [12]. To simulate the physiological situation, pCO_2 was retained close to 40 Torr by addition of 5.6% CO_2 in the gas mixture [13], and the measurement was carried out at a temperature of 37°C controlled by recycling water. The detailed measurement procedures were shown as follows.

(1) Firstly, 20 ml of the sample was introduced into the airproof-equilibrium apparatus.
(2) The initial gas mixture of O_2 and 5.6% CO_2 was then introduced through gas/inlet, with the apparatus slightly shaking until the oxygen partial pressure was shown over 140 mmHg by the oxygen meter, where the sample was saturated to 100% by O_2. Then, about 3 ml of the oxygenated sample was withdrawn by syringe, and then rapidly subject to UV-visible-near infrared spectrophotometer (175–3300 nm, Cary 5000, USA) to measure the optical absorption at 660 nm or 940 nm, which was taken as $(OD)_{oxyHb}$.
(3) Thereafter, the sample was gradually desaturated by using a new gas mixture containing 5.6% CO_2 in N_2. At predetermined oxygen pressure (P) intervals, the optical absorption of the sample was also tested at 660 nm or 940 nm, taken as $(OD)_{obs}$. During this stage, at least 5 given P and the corresponding different oxygen-saturated samples should be tested.
(4) Finally, the sample was completely deoxygenated and the absorption at 660 nm or 940 nm was referred to $(OD)_{deoxyHb}$. The sample should be tested under 100% to 0% O_2 saturation to obtain the oxygen dissociation curve.
(5) The value of Y at a given P was calculated according to the Eq. 1 [11].

$$Y = \left(OD_{obs} - OD_{deoxyHb}\right)/\left(OD_{oxyHb} - OD_{deoxyHb}\right) \quad (1)$$

(All the values of OD were generated in three independent experiments, and the optical absorption of HbP loaded Hb was subtracted by the baseline of blank HbP at 660 nm or 940 nm to eliminate the absorption of polymer matrix).
(6) The value P and the corresponding *Y* were taken as *X*-axis and *Y*-axis, respectively to fit oxygen dissociation curve.
(7) P_{50}, the O_2 pressure where Hb (or HbP) is half saturated with O_2, is directly obtained from ODC [14].

3 Results

3.1 Characterization of HbP

HbP, prepared by the modified solvent diffusion/evaporation double emulsion technique in our experiment, was accompanied with uniform spherical shape and an average diameter about 200 nm characterized by TEM image and size distribution (Fig. 2), indicating a potential of long-term circulation in blood stream [9, 15]. In addition, the smaller size of HbP, compared to RBCs, is advantageous in medical emergencies because it can pass through blockages, such as blood clots.

3.2 The ODC and P_{50}

The average optical absorption at 660 nm of the native BHb (column a) and PLA-PEG HbP (column b) under various P, and the corresponding Y calculated according to the Eq. 1 were presented in Table 1. The fitted sigmoid ODC and P_{50} of native BHb and HbP were shown in Fig. 3. The results revealed that the ODC of HbP was similar to that of native BHb. HbP was accompanied with P_{50} (26.1 mmHg), close to that of the native precursor protein BHb (27.3 mmHg) [16].

To further evaluate the reliability of the method, other biodegradable polymers such as PLA, PCL and PCL-PEG encapsulated HbPs were also investigated, and healthy human blood was used as control. The spectroscopic absorption of these polymeric nanoparticles or blood was also observed to be sensitive to O_2 pressure imposed (data

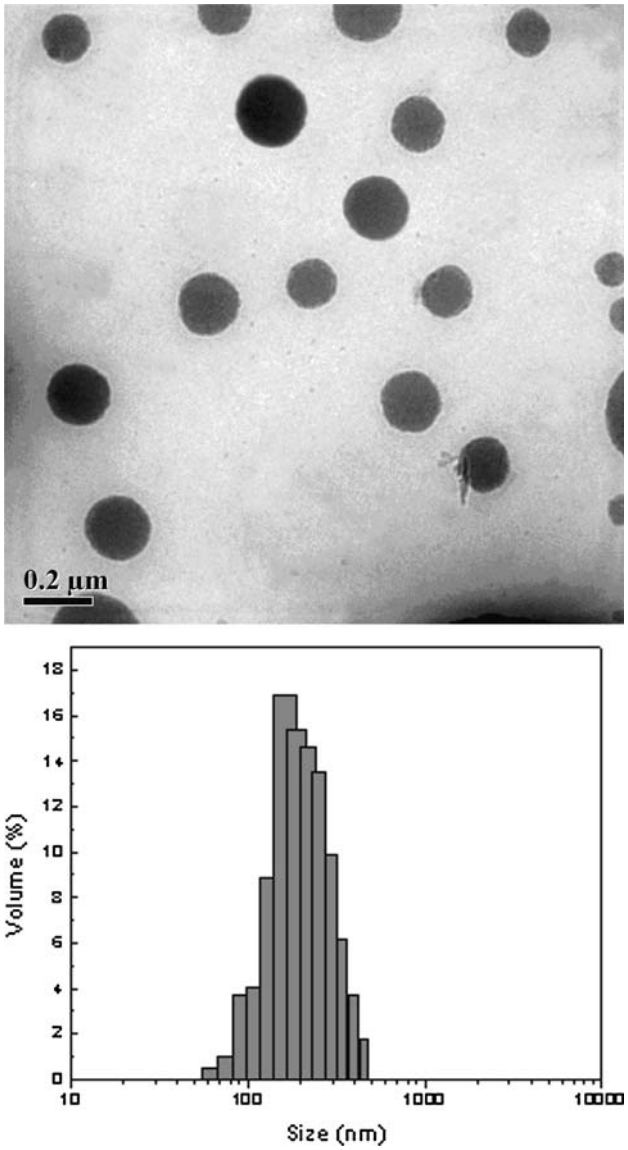

Fig. 2 TEM image and the size distribution of Hb-loaded nanoparticles (HbP)

not shown), and the obtained P_{50} values were listed in Table 2. It could be seen that the P_{50} of PLA, PCL and PCL-PEG HbPs were 25.4, 23.8 and 26.5 mmHg, respectively, with 26.8 mmHg for human blood.

4 Discussion

It is well known that the normal Hb can reversibly bind and release oxygen. At high P, Hb tends to bind O_2 to form gaseous ligand oxyHb, and deliver O_2 at O_2-poor environment to become nongaseous ligand according to the following equation: $HbO \rightleftharpoons Hb + O$. In general, the fraction of oxygenated Hb depends on the P imposed. In the meantime, oxyHb and deoxyHb show differential absorption spectra in visible light region. So the Hb with gaseous or nongaseous ligands can be determined by spectrophotometry, which relates the changes in absorption spectrum to the degree of saturation of Hb with ligands. Based on this,

Table 1 Changes in the average optical absorption, and the corresponding oxygen saturation (Y) of native Hb and HbP loaded-Hb with altered oxygen pressure (P) at 660 nm

P (mmHg)	(OD)[a]	Y (%)[a]	(OD)[b]	Y (%)[b]
140.00	0.764 ± 0.001	100.7 ± 4.2	1.103 ± 0.000	100.0 ± 0.0
120.00	0.763 ± 0.001	100.2 ± 3.3	1.092 ± 0.001	99.8 ± 3.2
110.00	0.762 ± 0.000	100.0 ± 0.0	1.103 ± 0.001	100.0 ± 2.7
100.00	0.762 ± 0.000	100 ± 0.0	1.102 ± 0.000	100.0 ± 0.0
82.08	0.753 ± 0.002	98.2 ± 7.8	1.078 ± 0.002	95.3 ± 7.2
68.60	0.733 ± 0.001	88.1 ± 3.4	1.070 ± 0.003	89.6 ± 2.9
40.50	0.703 ± 0.001	75.8 ± 3.6	1.031 ± 0.003	78.6 ± 8.8
26.20	0.636 ± 0.003	48.2 ± 8.7	0.946 ± 0.002	53.4 ± 6.7
19.20	0.604 ± 0.002	35.1 ± 7.8	0.903 ± 0.002	43.5 ± 7.7
14.40	0.576 ± 0.001	23.9 ± 2.4	0.865 ± 0.001	24.1 ± 8.6
0. 0.00	0.518 ± 0.001	0.0 ± 0.2	0.796 ± 0.001	0.0 ± 1.1

[a] Refers to native BHb

[b] Refers to Hb-loaded nanoparticles (HbP)

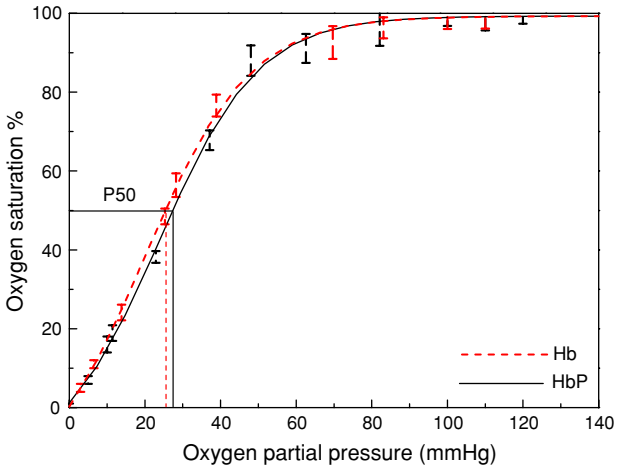

Fig. 3 Oxygen dissociation curves of native Hb (black solid line) and PLA-PEG HbP (red dash line) measured by the airproof-equilibrium apparatus under 37°C. Hb or HbP (50 uM, Hb) was resuspending in phosphate buffer (pH 7.4, 0.1 M Cl^{-1}). P_{50} of Hb and HbP were 26.1 mmHg and 27.3 mmHg, respectively SD

Table 2 P_{50} of different polymer entrapping materials entrapping hemoglobin HbP, measured by the noninvasive method, human blood used as control

Hb-loaded particles	P_{50} (mmHg)
Human blood	26.8 ± 0.1
PLA HbP	25.4 ± 0.2
PCL HbP	21.9 ± 0.1
PCL-PEG HbP	26.5 ± 0.3

Giardina et al. established a simple method in visible light region to measure the Y of cell-free Hb by a modified Thunberg tube in 1981 [11]. Hb, entrapped in polymer matrix, however, can not be directly detected by this method in visible light due to the barrier of the entrapping materials. To extract the entrapped Hb by some solvents is a possible method. Nevertheless, the treatment by organic solvents may influence the Hb molecules, such as oxidation or denaturation, which may make the measured Hb departure from the real state encapsulated.

Due to the strong penetrating property, NIRs is usually used as the light source in pulse oximeters. It was assumed that NIRs can penetrate the biodegradable polymer matrix to nondestructively detect the entrapped Hb. So, in this study, NIRs was used to determine the fraction of oxygenated Hb of the entrapped Hb. But two aspects should be taken into consideration: (1) NIRs can penetrate polymer matrix. (2) The binding of gaseous and nongaseous ligands to Hb entrapped in the polymer matrix should be response to NIRs.

With this respect, PLA-PEG HbP sample under the oxygenated (saturated 100% with O_2) and deoxygenated (deoxygenated with N_2) condition was scanned in the region of 600–1000 nm, and the absorption spectra subtracted by the corresponding blank HbP were presented in Fig. 4. The results demonstrated that oxygenated and deoxygenated HbP showed different optical absorption spectra in NIRs region, which confirmed that NIRs were able to penetrate the polymer matrix to detect the Hb encapsulated, because the different absorption spectra only originate from the different binding of gaseous and nongaseous ligands of Hb entrapped in the same HbP sample. Meanwhile, the different ligands to Hb showed different optical absorption in NIRs region, especially at the wavelength of 660 and 940 nm (Fig. 4), which was in agreement with the results described as Zijlstra [17]. It was reported that the wavelengths of 660 and 940 nm were usually employed in pulse oxymeters because oxyHb and deoxyHb absorb NIRs related to the concentration of these chromophores in the tissues. Any given sample oxygenation state would yield a unique intensity ratio between the two wavelengths [18]. Thus the two wavelengths were also selected in the present study. The results in Table 1 revealed that the optical absorption of the sample at 660 nm altered with the various P imposed, regardless of native BHb or BHb-loaded nanoparticles, which also evidenced that NIRs could penetrate the polymer matrix and detect the entrapped Hb, because various P only gave rise to various fractions of oxygenated Hb in HbP, and thereby alteration of optical absorption.

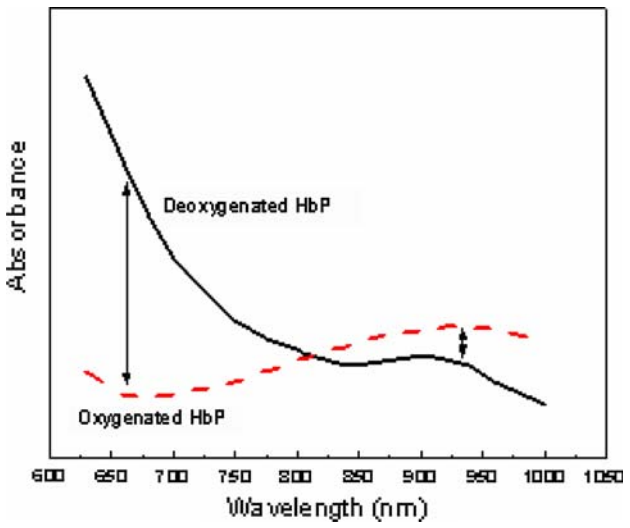

Fig. 4 Spectra of oxygenated and deoxygenated HbP in Near Infrared region Oxygenated and deoxygenated HbP show obvious differential absorption at wavelength of 660 and 940 nm

The ODC for whole blood correlates the Hb percentage saturation with O_2 to the partial pressure of the O_2. It describes how O_2 binds to Hb and how it is released, as can be imagined in the lungs and capillaries, respectively. The achieved ODCs of native BHb and HbP by the proposed method exhibited similar sigmoidal shape, and the values of P_{50} were also close, indicative of the validity of the method, as well as the retaining of the oxygen-carrying function of the entrapped Hb during the entire encapsulation process.

The optical absorption was observed to change with O_2 pressures, irrespective of HbPs or native human blood (data not shown). It is known that NIRs are successfully used to detect the Y of human tissues. The fact that the spectrophotometric absorption of human blood is response to O_2 pressure in the airproof-equilibrium apparatus, and the measured P_{50} (26.8 mmHg) (Table 2) is near to that of normal human blood, also verified the entire measurement was valid. Compared to PLA and PCL matrix, the P_{50} values of BHb entrapped in PLA-PEG and PCL-PEG exhibited more close to those of native BHb (Table 2), which may originate from the fact that polymer in itself set an effect on BHb molecules under the double emulsion treatment. PEGylated polymers may create a hydrophilic microenvironment within nanoparticles to protect the involved proteins due to the PEG segment with more hydrophilic nature [19]. BHb molecules may preferentially localize within deeper sections of the particles matrix to form core-loading, and thereby result in weaker interaction with hydrophobic matrix or minimize exposure to the solvents and fierce shear stress during the emulsification processes, which may throw a deleterious effect on the BHb molecules.

5 Conclusion

Based on the work of Giardina about the determination of the oxygen saturation of cell-free Hb, a simple approach

was established on the basis of the strong penetration of NIRs, as well as different absorption of oxyHb and deoxyHb in NIRs regions, i.e. 660 and 940 nm with the aid of the airproof-equilibrium apparatus. Despite of the strong penetrating ability of NIRs successfully employed in clinical practice or other situations, NIRs used in determining the oxygen binding-releasing capacity of artificial oxygen carriers have not yet been reported. In addition, the process did not destruct the polymeric nanoparticles, representing an accurate and nondestructive process. In view of the strong penetrating ability of NIRs, the method proposed has a potential to non-invasively analyze the drugs incorporated in the biodegradable polymers or other matrix by NIRs. At the same time, the method, in fact, was a simple and inexpensive alternative procedure for spectrophotometric determination the oxygen binding-releasing capacity of oxygen carriers, because high cost instruments are not always available in the average laboratory.

It is worth noting that, due to the increased formation of methemoglobin and a decline in 2,3-DPG content in whole blood, P_{50} might decrease with the time. Thereby, the blood sample should be tested immediately after blood collection in clinical practice. The same case occurs for the artificial oxygen carriers. It is recommended to store the sample in the ice bath until test. Furthermore, the same sample should be tested within 30 min to avoid the influence of methemoglobin formed.

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