J Mater Sci: Mater Med (2010) 21:241–249
DOI 10.1007/s10856-009-3864-4

Quantitative analysis of hemoglobin content in polymeric nanoparticles as blood substitutes using Fourier transform infrared spectroscopy

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Received: 17 November 2008 / Accepted: 26 August 2009 / Published online: 4 September 2009

Abstract Based on the penetrability of IR within the polymeric nanoparticles, a novel Fourier transform infrared spectroscopy (FTIR) method, with polyacrylonitrile (PAN) as the internal reference standard, was developed to quantify the hemoglobin (Hb) content in Hb-based polymeric nanoparticles (HbPN). The HbPN was fabricated by double emulsion method from poly(ethylene glycol)–poly(lactic acid)–poly(ethylene glycol) triblock copolymers. Depending on the characteristic un-overlapped IR absorbances at 1540 cm^{-1} of Hb (amide II) and at 2241 cm^{-1} of PAN (–C ≡ N), calibration equations, presenting the peak height ratio of Hb and PAN as a function of the weight ratio of Hb and PAN, were established. This new quantification method is validated and used to the determination Hb content in HbPN. Due to the good results of this calibration strategy, the proposed simple FTIR approach with minimal sample-needed and solvent-free makes it useful for routine analysis of protein content and could be also applied to any other drug/protein encapsulated particles.

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1 Introduction

The requirement for an artificial blood substitute continues to grow, especially because of the risk of contracting viral infections such as HIV or hepatitis C in nowadays [1]. A comprehensive study of incorporation of Hb in polymeric nanoparticles (HbPN) for preparing the blood substitute system has been undertaken. This is an efficiency strategy for the blood substitute preparation which mimic the structure of the native red blood cells (RBCs) to provide the ability to bind/deliver oxygen to tissues in body [2]. Therefore, the property of binding/delivering oxygen to tissues of HbPN is one of the most critical issues. It is well-known that Hb is composed of four polypeptide chains and each of them can bind one O_2 molecule via iron ion. Therefore, Hb loading in HbPN, to a great extent, determines the oxygen delivery/carrying capacity of HbPN and accordingly a valid method to evaluate Hb loading is also of great importance. However, just like other drug nanoparticles, the common methods for investigating the protein loading in encapsulated particles include two steps: firstly, the encapsulated protein is collected by extraction after particles shell being destroyed, filtration and followed by centrifugation. Secondly, the obtained protein is determined by HPLC [3–5] or UV-Vis [5, 6]. However, there is a hidden disadvantage to the protein loaded nanoparticles system even though this two-step method has been widely used to quantify the protein content for many years. Due to its easy denature under the strong acid [7] and alkali [8] circumstance, even under some common solvents [9], there is inevitably deviation of measured values from the truth to some extent. Therefore, it is clear that development of an alternative facile and precise strategy to investigate the protein level is greatly needed for the fabrication of encapsulated Hb-based blood substitutes with desirable oxygen-carrying capacity.

Owing to relatively easy and un-invasive to prepare samples in powder, bulk and thin film [10], Fourier transform infrared spectroscopy (FTIR) has been considered a powerful "in situ" analytical tool, which allows in a short time, to quantify the concentration of several specific chemical compounds [11, 12]. With respect of quantification, several analytical methodologies have been developed, such as external reference standard, internal reference standard and combining the above two [13]. Among them, the internal reference standard, which not only can compensate the shift of baseline spectrum causing by the changes of circumstance (such as the temperature, the humidity and the air pressure, etc.) and the instrumental variation, but also the intensity of specific peak comparison could be allowed to obtain from the IR spectrogram directly among multitude spectrum accurately [14], has been extensively applied for the quantification assay of FTIR. The feasibility of FTIR for qualitative analysis of structure and composition of polymeric nanoparticles has been reported in the previous investigations [15–17], which illustrated that the IR spectra could penetrate the polymeric nanoparticles and provide information related to the changes of specific functional groups directly [18], and so as to the chemical structure of organic [15–17] and inorganic [19–22] membranes. These results suggest the potentiality to quantify the protein in polymeric particles by FTIR. However, there is no reference available up to date.

Aiming to examine the possibility of using FTIR as an accurate method for quantifying Hb content in HbPN, in this study, the HbPN was fabricated by double emulsion method used poly(ethylene glycol)–poly(lactic acid)–poly (ethylene glycol)(PEG–PLA–PEG) triblock copolymers with different molecular weight and molar ratio of [LA] to [EG] as shell. Owing to the single and sharp IR absorbance at 2241 cm^{-1}, PAN was selected as an internal standard, which does not overlap with the IR absorptions derived from Hb and commonly used biodegradable polymer, such as polylactide (PLA) and its copolymers. Particularly, a given constant amount of polyacrylonitrile (PAN) was introduced to samples including the calibration standard samples and the detected samples in order to avoid eventual errors derived from environment and laboratory manipulation. In principle, this developed method is based on determination of the relative height of the characteristic absorbance peaks at 1540 cm^{-1} and 2241 cm^{-1} associated with the Amide II bending vibration band of Hb and the $-C\equiv N$ stretching vibration of PAN, respectively. Then, an equation correlating the peak height ratio of Hb and PAN with the weight ratio of Hb and PAN was established, with the assay evaluations including the linearity of standard curve, precision, recovery preformed. Based on this proposed method, the Hb loading in HbPN with different polymer composition was compared and discussed.

2 Experimental

2.1 Materials

Lyophilized bovine Hb was purchased from YuanJu Biotechnology Company (Shanghai, China). DL-Poly(L-lactide) (DL-PLA) (mw 40,000) and mPEG–PLA–mPEG triblock copolymers with molar ratio of [LA] to [EG] was 70–30, 85–15, and 95–5 were all synthesized by Jinan Daigang Biomaterial CO., Ltd.(Shandong, China). PAN was supplied by Nitrile department of Shanghai Petrochemical Company Ltd. Span80, Tween 80 and potassium bromides (S.P.) were all obtained from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China).

2.2 Methods

2.2.1 Preparation of the blank polymeric nanoparticles (BPN) and HbPN samples

HbPN were prepared by a multiple emulsion-solvent evaporation technique as described previously [23]. In brief, 0.5 ml aqueous solution of Hb (0.15 g/ml)was emulsified in the organic phase containing 10 mg polymer (oil phase) in methylene chloride with a probe sonicator (50 W for15 s) (JYD-900, Zhixin Instrument Co., Ltd., Shanghai, China) to form a primary water-in-oil emulsion. This initial emulsion was further mixed in an emulsifier-containing aqueous solution to make a $w_1/o/w_2$ double emulsion with the homogenizer. The double emulsion was then poured into 110 ml water solution, and the system was stirred for 4 h to completely remove the solvent through evaporation.

To figure out the effect of polymer composition on the Hb loading, pure PLA and copolymers with different LA/EG ratios (PEG-grafted degree with 5%, 15% and 30%, respectively) were applied to prepare HbPN using the identical preparation process, which named F1–F4 below respectively. The HbPN was collected by Lab Scale TFF system (Millipore Corporation, USA) and washed three times with distilled water before lyophilization.

To establish the standard curves for determining the Hb content in HbPN, the corresponding blank polymeric nanoparticles (BPN) were also prepared by the same multiple emulsion-solvent evaporation technique except with H_2O as the inner water phase instead of bovine Hb solution.

2.2.2 Characteristic of physicochemistry

Approximately 100 mg microparticles were re-dispersed in 10 ml phosphate buffer saline (PBS, pH 7.4) for several minutes using an ultrasonic bath. The size distributions and the zeta potential of the NPs suspension were determined at 25°C by Dynamic Light Scattering (DLS) using Zetasizer

Nano ZS (Malvern Instruments Ltd., UK) with an angle detection of 90°. Morphology of particles was observed by a transmission electron microscope (TEM, Hitachi H-7500, Japan).

2.2.3 Establishment of calibration standard curves

To determine the Hb content in HbPN with different molar ratio of PLA/PEG, the corresponding calibration standard curves were separately established. The standard samples consisting of Hb, PAN and BPN prepared from pure PLA or mPEG–PLA–mPEG (PEG-grafted with 5%, respectively) were accurately weighed amount according to the weight ratio shown in Table 1. In order to avoid the weighting error, we prepared the total weight of 2000 mg of calibration samples. The calibration curves were established over five analyte-levels in triplicate, respectively, and plotted between amounts ratio of Hb and PAN versus average response (peak height ratio of these two analytes).

To maintain the same light distance of propagation, all the samples were weight accurately with 2 mg and ground thoroughly into a fine powder with 198 mg (±0.1 mg) potassium bromide and pressed into disks (0.5 mm in thickness) with a total amount of 200 ± 0.1 mg.

2.3 FTIR measurement assay

The infrared absorbance spectra of Hb and PAN were simultaneously using NICOLET 5700 (Thermal Nicolet, USA) between 4000 and 400 cm^{-1}. 32 scans per sample were collected in absorbance mode at a spectral resolution of 4 cm^{-1}. All spectra were ratio against a background of potassium bromide spectrum under the same circumstance. The data were processed by omnic E.S.P software. The mixture pellets for determination were pressed on YP-2 tablet press machine (Shanyue Scientific Instrument Factory, Shanghai, China).

Table 1 The five ratio-points of calibration standards weighted carefully for the four linear regression curves

Samples[a]	1	2	3	4	5
Hb	1	1.25	1.5	3	6
BPN[b]	0.3	0.3	0.3	0.3	0.3
PAN[c]	6	6	6	6	6

[a] The calibration standard samples (Sample1–5) were the mixture of Hb–BPN–PAN according to the 5 levels of weight ratio(w/w/w)

[b] Biodegradable BPN were formulated by (F1) PLA, (F2–F4) mPEG–PLA–mPEG, PEG-grafted degree with 5%, 15% and 30%, respectively, and all weighted according to the same ratio above

[c] Internal standard PAN used for calibration curves establishment was added into the mixture for a constant weight

2.4 Assay evaluation–recovery and precision determination

The assay described above, developed to quantify free Hb, free PAN and Hb in nanoparticles, was validated by analysis of quality control methods [24]. Precision and accuracy were assessed by fulfilling replicate analyses and spotting recoveries of Hb content in nanoparticles against the above mentioned calibration curve, respectively.

2.4.1 Precision test

The precision of the method was evaluated by inter- and intra-day repeatability. The validation samples of each type of polymeric nanoparticles were analyzed in quintuplicate on more than three different days to determine intra- and inter-day precision with the percent relative standard deviation (R.S.D.) round the mean as the validation data of the low, medium and high Hb content in standard curves, respectively. The accepted intra-day precision should be within 5% and the inter-day should be less than 15% [24].

2.4.2 Spotting recoveries of Hb

The recovery experiment was performed as follows: accurately weighted the standard samples with low, medium and high level, having a constant internal standard concentration; and determined the Hb content which named Original Concentration. Thereafter, the sample was added pure Hb with the same weights as Original Weight and determined the Hb content again according to Section 2.3 after ground thoroughly, which named Measured Concentration. Theoretically, the Measured Concentration should be two folds of the Original Concentration. Therefore, the comparison of Measured Concentration and Original Concentration according to Eq. 1 is defined as recovery rate.

The sample recovery was defined as:

$$\text{Recovery}(\%) = \left(MC^{*}_{Hb} - OC^{**}_{Hb}\right)/OC_{Hb} \times 100\% \quad (1)$$

*, MC is the short of Measured Concentration; **, OC is the short of Original Concentration

The recoveries show the closeness of agreement between the observed value and the theoretical value after added the known amount analytes. The ideal recovery ranges should be between 95% and 105%, and the measured concentration should be in the linear range of linear regression equations.

2.5 Determination Hb loadings in HbPN

The Hb contents in HbPN with pure PLA or mPEG–PLA–mPEG were determined by the proposed FTIR method. The as-prepared HbPN and standard PAN were

Table 2 The actual weights of nanoparticles and PAN of each sample with the ratio be around 1:4

Samples[a]	F1	F2	F3	F4
W_{NP}(g)	0.0152 ± 0.0001	0.0151 ± 0.0002	0.0152 ± 0.0002	0.0151 ± 0.0003
W_{PAN}(g)[b]	0.0601 ± 0.0002	0.0603 ± 0.0002	0.0601 ± 0.0001	0.0602 ± 0.0001

[a] The samples F1 to F4 were prepared by biodegradable shell polymers: (F1) PLA, (F2–F4) mPEG–PLA–mPEG, PEG-grafted degree with 5%, 15% and 30%, respectively, using the identical preparation process

[b] Internal standard PAN used for quantitation analysis was added into the mixture for a constant weight

mixed in the ratio of 1:4 (as shown in Table 2). The mixture was grounded and homogenized thoroughly with potassium bromide into a fine powder with total amount of 200 ± 0.1 mg and pressed into disks with 0.5 mm around in thickness which was used to determine the Hb content according to Sect. 2.3 by FTIR.

3 Results and discussion

As one of the major physicochemical properties, drug loading mainly influences the therapy of nanoparticles. As mentioned as before, the Hb amount in HbPN, to a great extent, determines the oxygen delivery/carrying capacity of the nano-blood-substitute.

3.1 Physicochemistry characteristics of the HbPN

The physicochemical properties, including particle size, polydispersity index and the ζ potential of the HbPN derived from PEG–PLA–PEG triblock polymers with different compositions are presented in Table 3. It can be found that the composition of PEG–PLA–PEG triblock polymers had no obvious effect on the particle size and polydispersity index. But the surface electrical potential distribution of the HbPN varied pronouncedly with the content of PEG chains. All HbPN prepared were negatively charged. The ζ potential of the HbPN from PEG-containing copolymer increased obviously compared with that from pure PLA. Furthermore, the ζ potential monotonically increased with the increase of the PEG content in copolymer. The higher the PEG content, the lower the absolute value of ζ potential.

Table 3 Particles size and surface charge of hemoglobin-loaded polymeric nanoparticles encapsulated by four types of polymer

Samples	PEG content in copolymer (%)	Mean diameter (nm)	Polydispersity index	ζ potential (mV)
F1	0	207.7 ± 15.6	0.215	−37.67
F2	5	213.0 ± 8.3	0.203	−22.8
F3	15	209.9 ± 12.5	0.209	−13.6
F4	30	201.5 ± 4.9	0.198	−2.23

Figure 1 shows the typical TEM image of the HbPN encapsulated in this study. The HbPN appeared spherical with a size range of 70–220 nm, indicating a potential of long-term circulation in blood stream. It was in accordance with the size distribution determination (Table 3).

3.2 Selection of the internal standards and the characteristic peaks

As is well known, the liquid sample cell is available to ensure accurate absorbance for quantitative analysis in FTIR. Nevertheless, for the HbPN system, it is difficult to find a proper solvent to simultaneously dissolve both Hb and the polymeric shell. Considering the fact that the physical characteristics of HbPN and the internal standard PAN are all in powder-like state,the solid mixture of these compositions are directly investigated by FTIR in this study.

Generally speaking, the internal standard should satisfy the following requirements: firstly, the standard should have a typical strong absorbance peak in spectral region

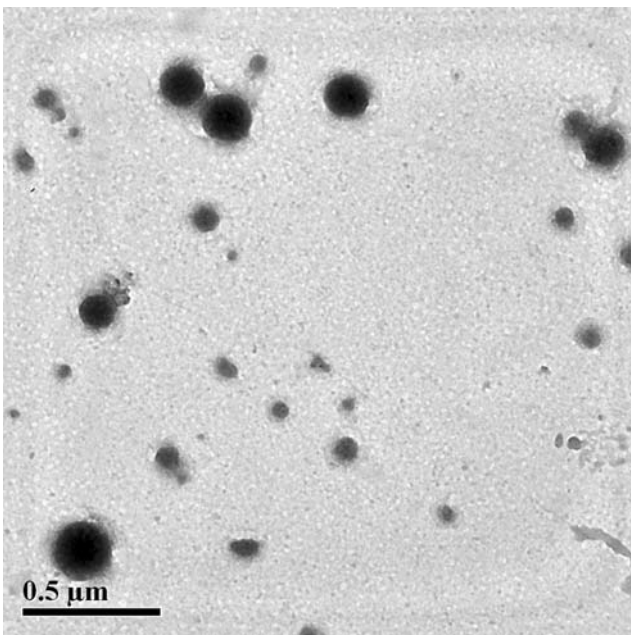

Fig. 1 TEM image of PEG–PLA–PEG (PEG5%) HbPN revealing shape and sizes of nanoparticles encapsulating hemoglobin molecules

which do not superpose the characteristic absorbency of the detected reagent. Secondly, the other chemicals in detected samples should not affect the characteristic peaks of the internal standard. Thirdly, the optional standard must be stable powder which should be inert to other compounds in the system.

To meet the above requirements, many commonly used internal standards had been investigated. Among them, PAN was considered to be the most suitable internal standard for our system. The full spectra of Hb, PAN, PLA and its copolymers used in this experiment in the wavelength regions from 2600 cm^{-1} to 1000 cm^{-1} are presented in Fig. 2. As shown in Fig. 2, just like other proteins, the prominent signals of Hb appeared at about 1655 cm^{-1} and 1540 cm^{-1} corresponding to the stretching vibrations of $–COO^-$ (amide I) and the bending vibration of N–H (amide II) [23] in pyrrole ring of protoporphyrin, which can be applied as universal probes for proteins because the peptidic amide bond is a basic and repetitive functional group in proteins [25]. In the case of PAN, the maximal absorbance peaks existed at 2241 and 1456 cm^{-1} (see Fig. 2), which could be attributed to the characteristic of stretching vibration of $–C\equiv N$ and deformation vibration of CH_2. To prevent the spectral interference, in this proposed method, the absorbance peaks of amide II band at 1540 cm^{-1} of Hb and stretching vibration of $–C\equiv N$ at 2241 cm^{-1} of PAN were in the possibility of being utilized to estimate the quantities of protein present in the samples. Furthermore, fortunately, from the IR spectroscopy of Fig. 2, there was also no interference spectrum derived from the PLA and its copolymers at 1540 cm^{-1} and 2241 cm^{-1}.

Additionally, the $W_1/O/W_2$ double emulsion technique, used in present article, is a classical process for drug/protein encapsulation. To obtain stable and homogeneously emulsification system, many surfactants, such as Tween-80 and Span-80, were introduced in the system. Then, an indispensable question was raised whether these emulsifiers affect the absorbance peaks of Hb and PAN, which was an important index for the feasibility of this developed FTIR method. To address this problem, the IR spectrum of Span-80, Tween-80 and the blank nanoparticles emulsified with Span-80 and Tween-80 were compared. As demonstrated in Fig. 3, Tween-80 had no absorbance around 1540 cm^{-1} and 2241 cm^{-1}, the pure Span-80 had a weak absorbance at 1562 cm^{-1}. But upon the forming of BPN, the spectral interferences derived from Span-80 almost disappeared. We believe that the Span-80 maybe eluted with the ultra-pure water during the collection procedure.

Based on these results, the selection of PAN as internal standard and the definition of specific absorbance peak enabled us to possibly quantify the hemoglobin content in nanoparticles.

3.3 The establishment of standard curves

The typical double emulsification-solvent evaporation process employed here to produce HbPN included 4 stepwise: (a) primary emulsification (w_1/o): Hb-containing aqueous phase was emulsified into an oil phase consisting of biodegradable polymer and surfactants, (b) double emulsification

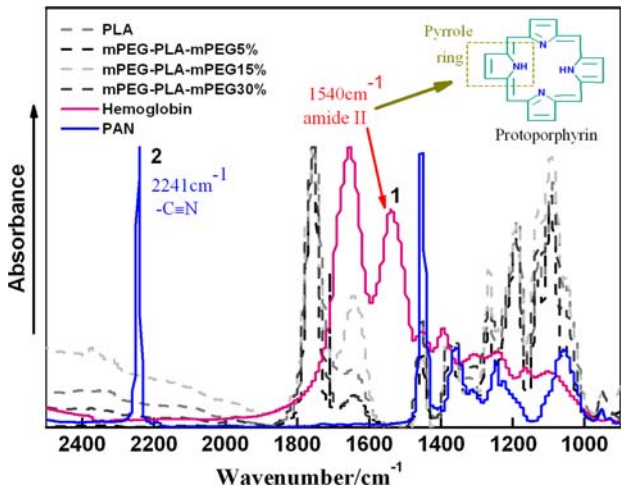

Fig. 2 FTIR spectra of PAN, Hb and the polymers used as nanoparticles shells. The special absorbance peaks in FTIR were (1) the bending vibration of N–H (amide II) at 1540 cm^{-1} in the pyrrole ring of protoporphyrin attributed to Hb and (2) the stretching vibration of $–C\equiv N$ at 2241 cm^{-1} attributed to PAN, respectively; the bands (1) Hb and (2) PAN used in this quantification method had no interference signals with the polymers used in experiments

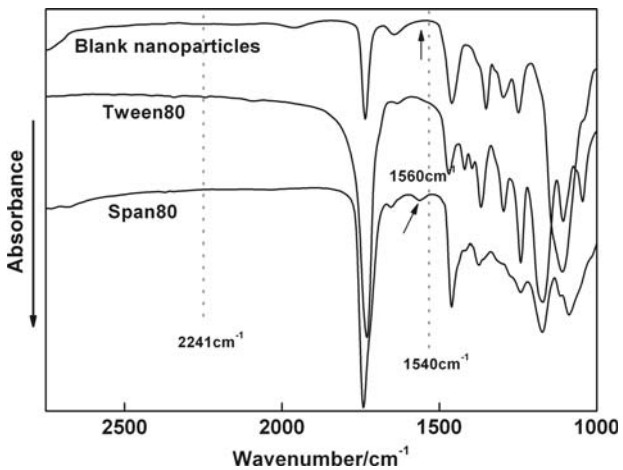

Fig. 3 Infrared spectra of Span-80 and Tween-80 comparing to blank nanoparticles encapsulated by Span-80 and Tween-80. There were no interference peak from Span-80 and Tween-80 at PAN special absorbance peaks 2241 cm^{-1}. Furthermore, note that the disappearance of the Span-80 absorbance peak at Hb special absorbance peaks 1560 cm^{-1} after nanoparticles formed which could verify the specificity of this promoted method

($w_1/o/w_2$): the primary emulsification was emulsified into a solution of the surfactant in water (external aqueous phase) to form a final $w_1/o/w_2$ double emulsion by high-speed mixing, (c) solidification: the organic solvent was removed by evaporation under atmospheric pressure at room temperature and then solid nanoparticles were formed, (d) purification: nanoparticles were isolated by centrifugation and purified through washing with distilled water before lyophilization. It became obviously that the basic scientific principle governing the nanoparticles forming was physical mixture without any chemical reaction. Subsequently, the freeze-drying and grind process would also not alter the components of nanoparticles. From this viewpoint, the HbPN can be considered as a mixture of Hb and BPN fabricated by the same polymer and process. Also, it is generally accepted that IR spectra can penetrate the polymeric nanoparticles and provide the information of the drug/protein ecapsulated. So, a series of Hb–BPN–PAN physical mixtures with different composition was used to establish the standard curves for Hb assay in HbPN. And the Hb–BPN–PAN mixtures were accurately weighed according to the weight ratios in Table 1.

From the FTIR spectra of Hb–BPN–PAN mixtures with different Hb contents and different encapsulation polymers within 3000–1000 cm^{-1} (not shown), it can be observed that the overall profile of the spectra was similar to each other. It can also be seen that in all cases, the peak height at 2241 cm^{-1} of PAN kept unchanging for all samples, whereas, with the increasing of Hb in the mixture, the band height at 1540 cm^{-1} corresponding to Hb monotonically increased. On the basis of the internal standard principle for FTIR spectroscopy, the standard curve generated by plotting peak heights versus concentrations was analyzed using linear regression method. Five level ratio-points of the absorbance of Hb (at 1540 cm^{-1}) to a baseline point of PAN(at 2241 cm^{-1}) corresponding to different weight ratio were used to demonstrate the calibration relation (Fig. 4). The calibration equations showed the good correlations between the spectral parameters and the samples compositions and the coefficient of determination R^2-value calculated was greater than 0.9994, indicating excellent correlation and reasonable accuracy [26]. These equations will be used to predict the Hb content of the polymeric nanoparticles samples according to Eq. 4 in Sect. 3.5 in the following experiment.

An interesting phenomenon was also observed in Fig. 4 that after being ground with BPN fabricated from different polymers, the special absorption at 1540 cm^{-1} of Hb was discriminating with different slope rates and different intercepts in the calibration curves. These results suggested that the hydrated PEG chains not only cover the outer shell of the nanoparticles to avoid agglomeration, but also influence the interaction between the polymer and hemoglobin.

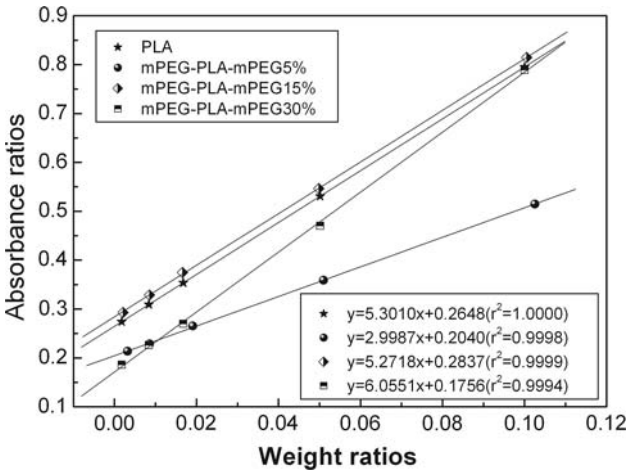

Fig. 4 Regression lines of polymeric Hb–BPN–PAN between the $h_{amide\ II}/h_{PAN}$ ratio (y) and the weight ratio of Hb to PAN (x). The calibration equations indicated to the PLA and mPEG–PLA–mPEG (PEG-grafted degree with 5%, 15% and 30%, respectively) polymeric nanoparticles fabricated by the identical preparation process

3.4 Assay evaluation

3.4.1 Linearity of the calibration curves

The calibration curves in Fig. 4 expressed the relationship between the absorbance heights ratio at 1540 cm^{-1} of Hb to a baseline point at 2241 cm^{-1} of PAN and the corresponding weight ratio of Hb and PAN. The confidence coefficient (R^2), a very important parameter of the calibration curve for verifying the reliability, was equal to or over 0.9994, indicative of a good linear correlation with excellent reliability within a range.

3.4.2 Recovery investigation

The accuracy (percent recovery) of the developed method for the determination of Hb content in HbPN mixture is investigated and the results are listed in Table 4. As it can be seen from Table 4, the amount of Hb added into each sample was within linear range mentioned in Fig. 4, and the recoveries of samples were all less than 5%, which was in agreement with the guidance for chemical: drug analytical procedures, methods validation and controls documentation [24].

3.4.3 Precision test

The overall method intra-day precision was determined by performing quintuplicate measurements on every sample with low, medium and high concentration at five different time-points within the same day, while inter-day precision was investigated on the same samples for four separate days according to the identical analysis method [27]. In

Table 4 Accuracy (percent recovery) of the method for the determination of Hb content in Hb–BPN mixture ($n = 5$)

Samples[a]	concentration	Theoretical hemoglobin amount (g)	Measured hemoglobin amount (g)	Analytical recovery (%)	Average recovery ± relative standard deviation (%)
F1	Low	0.0105 ± 0.0003	0.0104 ± 0.0002	98.41	99.37 ± 1.98
	Medium	0.0155 ± 0.0002	0.0152 ± 0.0005	98.06	
	High	0.0303 ± 0.0001	0.0308 ± 0.0006	101.65	
F2	Low	0.0105 ± 0.0002	0.0108 ± 0.0003	102.86	98.68 ± 3.88
	Medium	0.0155 ± 0.0003	0.0158 ± 0.0005	95.18	
	High	0.0301 ± 0.0001	0.0395 ± 0.0009	98.01	
F3	Low	0.0105 ± 0.0002	0.0101 ± 0.0003	95.23	97.22 ± 2.04
	Medium	0.0155 ± 0.0002	0.0152 ± 0.0006	97.13	
	High	0.0292 ± 0.0001	0.0290 ± 0.0004	99.30	
F4	Low	0.0103 ± 0.0002	0.0103 ± 0.0002	100.00	98.59 ± 1.65
	Medium	0.0155 ± 0.0001	0.0150 ± 0.0003	96.77	
	High	0.0303 ± 0.0002	0.0300 ± 0.0001	99.01	

[a] The samples F1–F4 were prepared by the degradable shell polymers: (F1) PLA, (F2–F4) mPEG–PLA–mPEG (PEG-grafted degree with 5%, 15% and 30%, respectively) by the identical preparation process

addition, the average precision with percent relative standard deviation (RSD%) for each sample was calculated (Table 5).

Obviously, the relative standard deviations of intra-day precision were all lower than 5%; in the case of the inter-day precision, the data were all lower than 15%, which were all acceptable for the FTIR assay [24]. Considering the storage of HbPN, there were many factors which could probably affect the final assay results: for example, in spite of samples being stored in desiccator at 4°C, the loss of water would inevitable happen which could result in weight loss. Besides that above, the losses in samples detection and the different circumstance in the different days would also be the reasons for the high deviation of inter-day assay. The relative standard deviations of the intra-day precision were low, indicating that the sample preparation and subsequent FTIR analysis were very reproducible [28].

In short, this assay complied with proposed drug analytical guidelines [24] for the validation of quantified method in terms of linearity, precision, accuracy of the method. Therefore, the linear curves within the linear ranges and the correlations made them suitable for quantification. Therefore, this alternative strategy can be applied to investigate the protein level in nanoparticles, especially for Hb.

Table 5 The intra- and inter-day precision (relative standard deviation) of the method for the determination of Hb content in Hb–BPN mixture (percent of drug loading)

Hemoblogin amount in mixture of Hb–BPN–PAN[a] (g)		Intra-day ($n = 5$) (<5%) Precision	Inter-day ($n = 4$) (<15%)Precision
F1	0.0201 ± 0.0003	2.07	4.41
	0.0303 ± 0.0001	1.78	3.63
	0.0401 ± 0.0001	1.67	2.63
F2	0.0201 ± 0.0002	1.99	4.57
	0.0301 ± 0.0001	1.58	3.32
	0.0401 ± 0.0003	1.44	3.09
F3	0.0200 ± 0.0002	1.93	3.77
	0.0292 ± 0.0001	1.88	1.61
	0.0400 ± 0.0001	1.15	3.81
F4	0.0200 ± 0.0002	1.79	1.45
	0.0303 ± 0.0002	1.62	2.03
	0.0401 ± 0.0002	2.71	3.46

[a] The samples nanoparticles F1–F4 were prepared by the degradable shell polymers: (F1) PLA, (F2–F4) mPEG–PLA–mPEG (PEG-grafted degree with 5%, 15% and 30%, respectively) by the identical preparation process

3.5 Determination of the Hb loaded in HbPN

In terms of the principle of internal reference standard, the concentration ratio of the detected protein to internal standard ($x_p/x_{i.s.}$ for short) exhibits a linear relationship with the adsorption peak height ratio of protein h_p to internal standard by the equation below:

$$x_p/x_{i.s.} = (h_p)/(h_{i.s.}) \times A + B \quad (2)$$

A and B represent the slope rate and intercept of the standard curves, which can be obtained from the standard curves.

Additionally, owing to the mixture samples in present study were ground and homogenized thoroughly before determination, the weight ratios of protein to internal standard in determined-pallets could exactly represent the weight ratios of protein to internal standard $\left(W_p/W_{i.s.}\right)$ in the samples, as shown in Eq. 3. Accordingly, with respect to protein loading nanoparticles system, the protein content can be easily calculated by following Eq. 4:

$$\left(\frac{W_p}{W_{i.s.}}\right)_{sample} = \left(\frac{W_p}{W_{i.s.}}\right)_{pellet} = \left(\frac{x_p}{x_{is}}\right)_{pellet} = \frac{\left(\frac{h_p}{h_{i.s.}}\right) - B}{A} \quad (3)$$

$$\begin{aligned} D.L.\% &= \frac{W_p}{W_{NP}} \times 100\% \\ &= \frac{\left(\frac{h_p}{h_{i.s.}} - B\right)}{A} \times \frac{W_{i.s.}}{W_{NP}} \times 100\% \\ &= \psi \times \left(\frac{h_p}{h_{i.s.}} - B\right) \times 100\% \end{aligned} \quad (4)$$

In the present work, the Hb loading in HbPN prepared from pure PLA or mPEG–PLA–mPEG, (PEG-grafted degree with 5%, 15% and 30%, respectively) were investigated by FTIR with PAN as the internal standards and subsequently calculated from above Eq. 4 respectively; and the results are presented in Fig. 5. It can be seen that the Hb loading almost remained constant when PEG percentage was lower than 5%. Whereas, when the PEG percentage in polymer increased from 5% to 15%, 10% improvement of the drug loading capacity in HbPN was observed. However, too high PEGylation percent was found to hamper the drug loading capacity. When PEG content was greater than 15%, the Hb loading decreased sharply with the increasing of the percentage of PEG conversely, indicating a typical PEG-dependent manner. These results are very interesting. In the previous studies, many investigators have reported the role of PEG molecular weight on the physical-chemistry characteristic as encapsulation efficiency and drug loading [29, 30], and so on. But it has received less focus on the different PEG content in triblock copolymers effect on the drug loading capability. The relationship between PEG percent and the drug loading here was fulfilled to provide a reference to the other studies to some extent.

As a whole, the method presented here provided a useful and convenience tool for the rapidly and accurately quantification of Hb in HbPN. Such spectroscopic analytical method presented several advantages over existing technologies such as simplified and facile procedures, reduced assay time, and lower consumption of samples and solvent-free. The possibility of predicting Hb concentration in HbPN is an invaluable tool because it is one of the most important parameters for the fabrication of HbPN and other oxygen carrier. In the future, this method can be extended to other drug/protein nanoparticles system, such as antitumor drug carriers [31] and other functionalized microcarriers [32, 33], if only the specific absorbance peaks of the drug/protein and the internal standard were selected. Moreover, the data presented in this work proved that the use of PAN as an internal standard was preferable and successful.

In fact, it is important to know there are some other necessary steps should be fulfilled before quantitative measurement to get these stable and accurate results. Firstly, the validation samples should be analyzed in no less than quintuplicate. For our nanoparticles system, the content of the drug in particles was inevitably affected by the process of microencapsulation which was, however, sensitive to circumstance [34–36]. On the other hand, the Hb loaded nanoparticles and internal standard would better to be weighted carefully and ground thoroughly into a fine powder. Otherwise, there would result in a large deviation from the 'real' value.

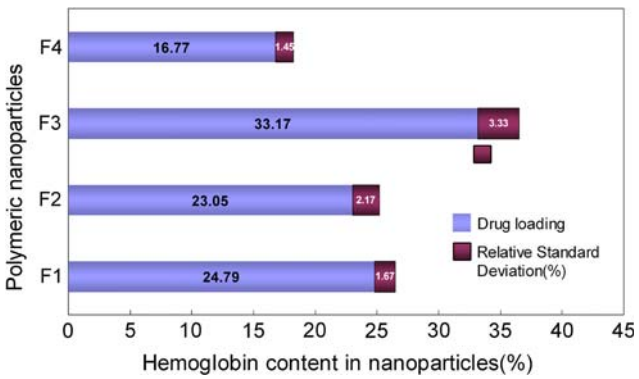

Fig. 5 Drug loading and relative standard deviation in HbPN. The samples F1 was prepared by PLA. The samples F2–F4 were prepared by copolymer PEG–PLA–PEG and the PEG percentage in copolymer were 5%, 15% and 30%

4 Conclusions

An FTIR spectroscopic method with internal standard reference has been developed for the determination of the Hb content in HbPN samples, which avoids the problem associated with the conventional method utilized in biomedical materials. This approach is based on the stand alone relationship between the absorbance peak strength or height and the concentration of the absorbing species, and the measurement of the integrated signal in windows obtained from the IR penetration signals through the samples that are typical of Hb and PAN coupled with systematic data processing. Imposed by perfect linear correlation, acceptable precisions and recoveries within linear ranges, this method was applied for quantitative investigation successfully. The consequence of drug loading determination indicated that the drug encapsulation capability had a typical PEG-dependent manner. Our new strategy should be helpful for the future polymeric nanoparticles as drug content exploitation.

Acknowledgements The authors acknowledge the financial support from the National High Technology Research and Development Program of China (863 program) (No. 2004AA-302050) and from Shanghai Nanotechnology Special Foundation (No. 0452nm022).

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