J Fluoresc (2009) 19:277–284
DOI 10.1007/s10895-008-0413-3

ORIGINAL PAPER

Optimization of the Near-Infrared Fluorescence Labeling for In Vivo Monitoring of a Protein Drug Distribution in Animal Model

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Received: 20 May 2008 / Accepted: 11 August 2008 / Published online: 29 August 2008

Abstract The objective of this study is to optimize the parameters in labeling near-infrared (NIR)fluorescent dye cypate to protein drugs for in vivo optical imaging of drug distributions in animal model. L-ASparaginase (L-ASNase) was used as a protein drug model for the study. To achieve this goal, various labeling conditions, including different catalysts, feed ratios of all components, pH conditions, temperatures, and reacting durations, were investigated. The dye-to-protein (D/P) ratio and enzymatic activity were designated as the metric to evaluate the labeling process. The stability of the cypate–protein conjugate in blood serum and its distribution in small animals were subsequently inspected. Results showed that feed ratio of L-ASNase and the pH value played the most important role in adjusting the labeling efficiency. Reaction duration and temperature had less effect on the dye-to-protein labeling properties. The optimal condition for the labeling of cypate to L-ASNase was 4 h reaction duration at 4 °C and pH 8.5 under catalysis by HOBt/HBTU. The dynamic distribution in animal model displayed that the labeled L-ASNase firstly accumulated in liver and cleared from the enteron system. This study demonstrated that the NIR image system combined with NIR probe has the capability to trace the dynamics of protein drugs in animals for drug development.

Keywords Near infrared · Fluorescence dye · Protein drugs · Labeling condition · In vivo optical imaging

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Introduction

Characterized with the virtue of high physiological activity, specificity, and low toxicity, protein drugs are becoming mainstream therapeutic agents and constitute a substantial portion of the compounds under preclinical and clinical development in the global pharmaceutical industry [1–3]. Consequently, the in vivo evaluation of drug properties, such as drug bio-distribution, pharmacokinetics, pharmacodynamics, becomes crucial in the protein drug development. Previously, a number of analytical techniques, such as immunoassay, bioassay, and isotope labeling, were exploited to analyze the protein drug process within the experimental subjects [4, 5]. However, all the above techniques, except isotope labeling, are unable to perform the in vivo measurement of protein drug in biological matrices. They require the tedious and intricate process of sample preparation such as sample collection and purification. Furthermore, the clearance of protein drugs from the specific tissue/organ is fast and the above analytical techniques cannot catch up with its dynamic changes. Therefore, a non-invasive in vivo real time monitoring modality for protein drugs in biological matrices will timely meet the needs and greatly expedite the step of protein drug development.

Near-infrared light (NIR; 700–900 nm), characterized by the deeper tissue penetration as well as non-ionizing and

non-radioactive radiation, provides a non-invasive techniques for in vivo real time monitoring/tracing of biological signals in living tissues [6–9]. However, most of the protein drugs lack unique optical properties within the NIR range, making it necessary to label them with an extrinsic NIR fluorescence dye for in vivo study [6, 7, 10]. Thus, the successful labeling of extrinsic NIR probe to protein drugs becomes the keystone for their in vivo evaluations. Many applications regarding to the labeling of NIR probe to macromolecules, such as antibodies, antibody fragments, proteins, peptides and so on, were reported [11–13]. However, only limited efforts have been devoted to the labeling conditions for better labeling efficiency and higher pharmaceutical/enzymatic activity, which is the most important factor for protein drug development.

In this study, we investigated the optimal parameters in the labeling processes. Here, cypate, a derivative of ICG, was synthesized by Achilefu's group and applied to label protein drug for in vivo tracing [14–18]. L-ASNase was used as protein drug model for optimization of the labeling condition. L-ASNase is a remarkably effective therapy protein drug for those specific cases where blood cells become cancerous, such as in acute lymphoblastic leukemia (ALL) and in non-Hodgkin's lymphoma (NHL) [19, 20]. To optimize protein labeling and biological activity of the enzyme, different reaction conditions, such as catalyzing system, feed ratio of two components, pH condition, temperature and reaction duration, were investigated in this study to get better D/P ratio and higher conjugate activity. Finally, the dynamic distributions of protein drugs were monitored by a home-built NIR reflectance imaging system.

Materials and methods

Chemicals

The NIR fluorescent dye cypate ($C_{41}H_{41}N_2O_2$) was synthesized by Dr. Achilefu's group. It was sealed in dry atmosphere to prevent hydrolysis during storage. L-ASNase with a molecular weight of 138.4 kDa was provided by Qianhong Bio-pharma Co., Ltd (Changzhou, Jiangsu, China). Three catalysts, *N*,*N*′-Dicyclohexyl-carbodiimide (DCC), 1-Hydroxy-1H-benzotriazole, monohydrate (HOBt) and *O*-Benzotriazole-*N*,*N*,*N*′,N′-tetramethyluronium-hexafluorophosphate (HBTU), were purchased from GL Biochem Co. Ltd (Shanghai, China) and used without further purification. The Nessler's reagent was prepared by mixing KI, HgI_2 and 25% NaOH together and stored in a brown flask at room temperature. All chemicals were of analytical reagent grade unless otherwise mentioned.

Apparatus

A UV/vis Spectrophotometer (JH 754PC, Shanghai, China) was used to perform the absorbance measurements. PHS-25 pH meter (Shanghai, China) was used to measure the pH values. DYY-8C Electrophoresis power supply and DYCZ-24D electrophoresis chamber (Beijing, China) were used in SDS-PAGE protein analysis.

A self-built NIR reflectance imaging system is composed of an excitation laser (λ=765.9 nm, NL-FC-2.0-763), a high sensitivity NIR CCD camera (PIXIS 512B, Princeton Instrument) and an 800nm long pass filter for capturing the emitted fluorescence from the tissue. This system was used to image the distribution of cypate-labeled protein drug in real time. In addition, another laser (λ=808 nm, HLU32F400-808, LIMO Co. Ltd) is supplied as background light to obtain the profile of the subjected animal. Otherwise, the imaging was completely black except the fluorescence spots, which made it difficult to recognize the different regions of the animals.

Labeling L-Asparaginase with cypate

The dye cypate was firstly dissolved in DMSO and the fresh cypate solution (1 mg/ml) was then mixed with catalysts for activation of carboxylic aid functional group. To obtain better labeling efficiency, three catalyzing combinations, DCC/HOBt, EDC/HOBt and HOBt/HBTU, were investigated in the labeling processes, respectively.

After activation of cypate by catalyzing combinations for about 2 h, a specific amount of L-ASNase sample was dissolved in buffer solutions and then incubated with the active cypate solution. The mixture was stirred at a constant rate. After several hours' reaction, the dye–protein combination was achieved and separated from the free dye by dialysis against deionized water. The desired dye-labeled protein was collected in bag filters. To obtain an optimal practice for enzyme labeling, different reacting conditions, such as the feed ratio of the two components, pH condition, temperature, reacting duration, were investigated. In the whole experimental procedure, cypate solution was kept in dark to minimize photo-instability, and the concentration of L-ASNase used for conjugation was 1 mg/ml except in the feed ratio optimization.

Characterization of labeling efficiency and enzyme activity

Labeling efficiency was characterized by the D/P ratio. Cypate has maximum absorbance at 788 nm and fluorescence at 830 nm, far away from the maximum absorbance of the protein. The peak data at 780 nm for absorption can be used to quantify the content of dye in labeling combination based on the standard curve of free dye. The

protein concentration was determined by the Bradford method, which showed no interference by cypate. The ratio of their molar concentrations was defined as the molecular D/P ratio.

The enzymatic activity of L-ASNase was considered to have the highest priority in the process of optimizing the labeling condition and was evaluated by the Nessler's reagent photometric method that was modified by Liu's group [21]. In brief, L-ASNase solution was firstly reacted with L-Asn solution at 37 °C for 15 min then stopped by 50% trichloroacetic acid. After centrifugation the supernatant was added to Nessler's solution and the absorbance was measured at 500 nm. Following this method, the labeled and original enzyme activities were determined respectively, and their ratio was defined as relative enzyme activity.

Furthermore, electrophoresis was used to assay the integrality of L-ASNase and sephadex-G100 gel chromatography was used to confirm the covalent conjugation of dye-to-ASNase.

Evaluation of the stability of the labeled protein drug in PBS buffer and blood serum

The same amount of non-labeled L-ASNase and the cypate conjugation were hydrated with 20% DMSO in 1 mL PBS and incubated at 37±0.1 °C. Aliquots taken at intervals were collected and immediately assayed by Nessler's method.

After dissolving in 100 μl 20% DMSO, each of these two samples was added to 900 μl blood serum, which was then incubated at 37±0.1 °C. Enzyme activity was detected by following the method mentioned above.

Imaging of the dynamics of protein drug in mouse model

The Kunming mice (4–6 weeks old, at weight of about 20–25 g) were purchased from Laboratory Animal Resources of China Pharmaceutical University. All experiments were carried out in compliance with the Animal Management Rules of the Ministry of Health of the People's Republic of China (document no. 55, 2001) and the guidelines for the Care and Use of Laboratory Animals of China Pharmaceutical University.

The Kunming mice were fasted one day before the experiment with enough water supply. For imaging experiments, 5 of the mice were anesthetized by 1 g/kg ethyl carbamate and placed in a prone position, with temperature kept at 37 °C. The mouse hairs were removed to perform better measurement for optical signals. One hundred micrograms of freshly labeled L-ASNase was administrated into each mouse through tail vein injection. A reflectance NIR imaging system furnished with a long pass filter in front of CCD camera was used to in vivo real time monitor the dynamic distribution of dye-labeled L-ASNase. The fluorescence images were taken at the designed time intervals by NIR imaging system adjusted at the appropriate gain and exposure time (0.2 s) of the CCD camera.

Results and discussion

Effect of catalyzing system on labeling process

The commonly used functional group for labeling protein is the primary amino group provided by the ε-amino group of lysine or the N-terminus. In this trial, the primary amino groups were linked to carboxy groups from cypate by amide bonds which are widely used in bio-conjugation for its high solution stability and biocompatibility. After evaluating the highly reactive coupling reagents, three catalyzing combinations, DCC/HOBt, EDC/HOBt and HOBt/HBTU, were investigated in L-ASNase labeling, respectively. Result showed that only HOBt/HBTU catalyzing system facilitated the covalent conjugate of cypate to L-ASNase, with the green solution observed in bag filters after dialysis. The covalent conjugate was confirmed by the sephadex G-100 gel chromatography. After dialysis, the rude dye-to-protein conjugate was subjected to gel filtration on a sephadex G-100 column. The sequent fractions of the elution were collected at a time course and the absorptions at protein wavelength and cypate wavelength were measured, respectively. The time profiles of the collected fractions are shown in Fig. 1.

Two protein absorption peaks were detected at around the 35th and 45th fractions of the elution, which indicated the existence of L-ASNase at around the fractions of 35th and 45th fractions. And meantime, at around the 35th

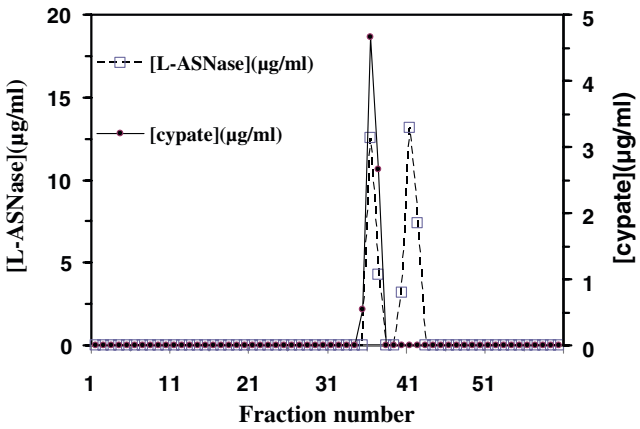

Fig. 1 The elution profile of rude CY-ASP by gel filtration on Sephadex G-100 column. Fractions collected from the column were assayed for proteins content by the bicinchoninic acid (*BCA*) Method and cypate content as described in "Materials and methods"

factions, cypate contents were also detected at the same time, which implied cypate existed at around 35th fraction. The protein peak and cypate peak appeared at the same time (at around the 35th fractions) demonstrated the conjugation of L-ASNase with cypate at around the 35th fractions. The 45th peak implied the unconjugated L-ASNase. The pure cypate peak was appeared at around the 100th fraction (date did not show here). The analysis from sephadex G-100 gel chromatography indicated the rude dye- protein reaction solution included three components, that is, conjugated cypate–ASNase, pure cypate and L-ASNase. The desired conjugation can be achieved after the sephadex G-100 gel separation. In contrast, DCC/HOBT and EDC/HOBT catalyzing combination were unable to catalyze their reaction as most of cypate converted to the powder form in the proceeding of these two reactions. The aggregated cypate made itself difficult to get close to L-ASNase and label them in aqueous solution. Thus, the HOBT/HBTU was used as an optimal catalyzing system in all the subsequent labeling synthesis. Although one L-ASNase molecule provided over 80 lysines for conjugating, the real D/P ratio is much lower as the dye hardly close up to every conjugating site due to the sterical structure.

Effect of pH condition on labeling properties

Figure 2 showed the influence of pH conditions on the D/P ratio and relative enzymatic activity. Results indicated that D/P ratio elevated by the increase of pH value, while there is an optimal condition (pH=8.5) existed for the relative enzymatic activity. Usually, L-ASNase possesses the highest stability and activity at pH 8.5. Reaction at this condition hardly did harm to its property. And thus, the highest enzyme activity of the conjugate appeared in the

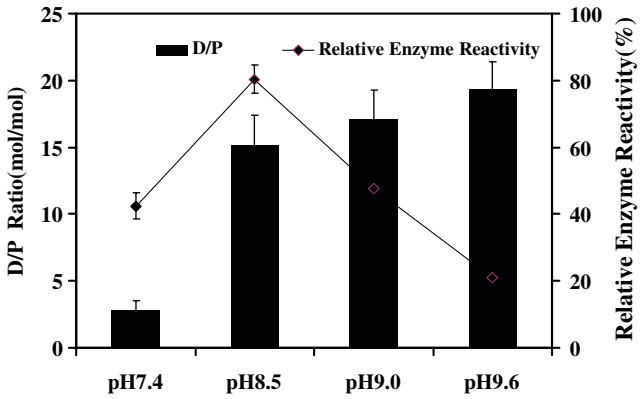

Fig. 2 D/P ratio and relative enzyme activity of L-ASNase labeled by cypate in different pH values (n=5). D/P ratio equals to the concentration ratio of dye-to-protein, and the relative enzyme activity is the retaining enzyme activity of labeled L-ASNase compared with unlabeled one. Both indicators mean the same in the following figures

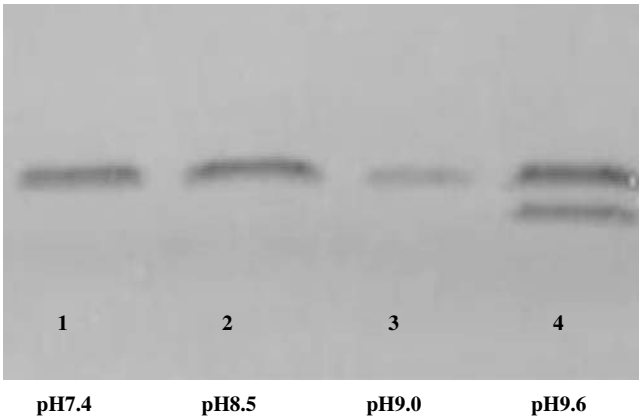

Fig. 3 SDS-PAGE electrophoresis of cypate-ASNase obtained at different pH values

pH 8.5 condition. At higher pH condition, there are many free basic radicals of protein existed in the buffer. And thus, the more dyes would bind to the protein drug, which result in the higher D/P ratio in the higher pH condition. However, the enzyme activity dropped quickly with the increase of the pH value. The reason is that the higher pH condition would facilitate decompose of the protein drug and render a lower enzymatic activity. This phenomenon was confirmed by the SDS-PAGE electrophoresis analysis, as shown in Fig. 3. Electrophoresis demonstrated that the dye-protein conjugation decomposed at higher pH (pH=9.6). As the enzymatic activity was the priority metric for evaluating the labeling properties, pH 8.5 (with highest enzymatic activity and reasonable D/P ratio) was determined as the optimal pH condition.

Effect of reaction duration on labeling properties

Compared to pH condition, reaction duration had less effect on both the D/P ratio and enzyme activity, as shown in Fig. 4. D/P ratio had a variation from 17 to 20 when reaction duration changed from 1 to 8 h, lower D/P ratios appeared

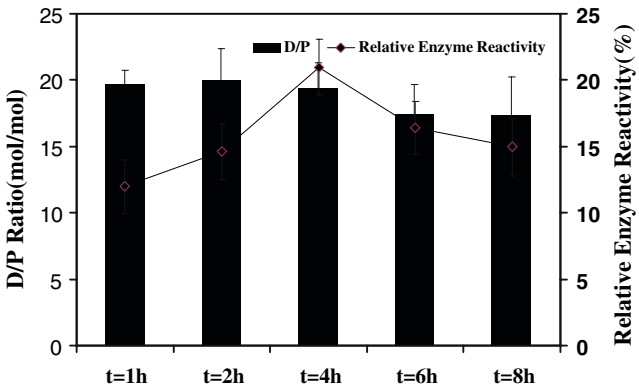

Fig. 4 D/P ratio and relative enzyme activity of L-ASNase reacted with reactive cypate for different duration times (n=5)

at the later stage (t=6 and 8 h). Although enzyme activity was affected more by the reaction time than that of D/P ratio, its fluctuation limited from 13% to 21%. An optimal reaction time (4 h) was identified for the highest relative enzyme activity.

In the reaction process, both synthesis and hydrolysis simultaneously existed all the time. The synthesis dominated the reaction at the early stage and hydrolysis took over the dominated place several hours later. Thus some of the conjugated compound were hydrolyzed in the later stage (t=6, 8 h) and led to lower D/P ratio and enzymatic activity (Fig. 4).

Effect of temperature on labeling properties

Figure 5 displayed the influence of temperature on the D/P ratio and relative enzyme activity. As expected, the enzyme activity decreased quickly with the increase of temperature when temperature was higher than 28 °C. This is due to the fact that higher temperature usually results in the change of protein conformation and disable active center of enzyme, inducing the decrease of the relative enzyme activity. Further, enzyme solutions usually keep their activity best at 4 °C. For D/P ratio, Fig. 5 implied a maximal value existed at 4 °C. Thus, carrying out the labeling reaction at 4 °C provided the optimal temperature for both D/P ratio and relative enzyme activity.

Effect of protein feed ratio on labeling properties

The effect of protein concentration on the conjugation efficiency was also investigated, which was shown in Fig. 6. The same amount of excess dye was applied in all the reaction. Results implied that D/P ratio decreased rapidly with increase of L-ASNase. The opposite phenomenon was depicted for enzymatic activity, where the relative enzyme activity elevated with the increase of L-ASNase. Both D/P ratio and enzyme activity were strongly affected by protein concentration.

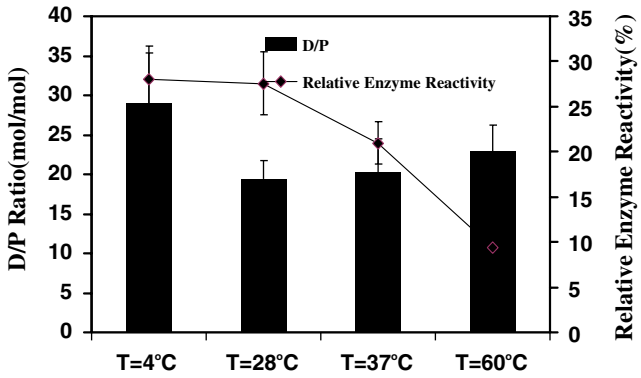

Fig. 5 D/P ratio and relative enzyme activity of L-ASNase labeled by cypate at different reaction temperatures (n=5)

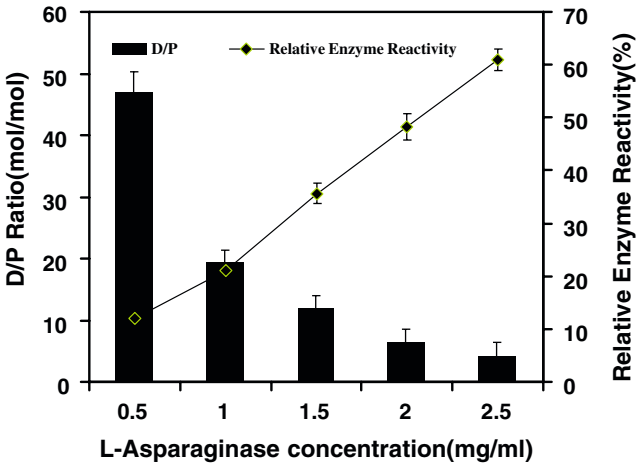

Fig. 6 D/P ratio and relative enzyme activity of progressive L-ASNase labeled by equal cypate (n=5)

Obviously, lower primary labeling ratio corresponded to less fluorescent molecule per protein. Maximal feed ratio of dye to protein (0.5 mg/ml) yielded the maximal labeling ratio (D/P ratio), as shown in Fig. 6. In contrast, the lower D/P ratio (higher protein concentration) in the reaction solutions had less effect on the structure of L-ASNase, thus a higher enzymatic activity was displayed at higher enzyme concentration. The observations were reasonable and theoretical expected.

Although higher D/P ratio could increase the detecting sensitivity for in vivo measurement, the high enzyme activity was the priority for a better therapeutic outcome. That means that the D/P ratio could be adjusted to the lowest level to keep the enzyme activity for better therapeutical efficacy. In this study, the optimal concentration of L-ASNase at

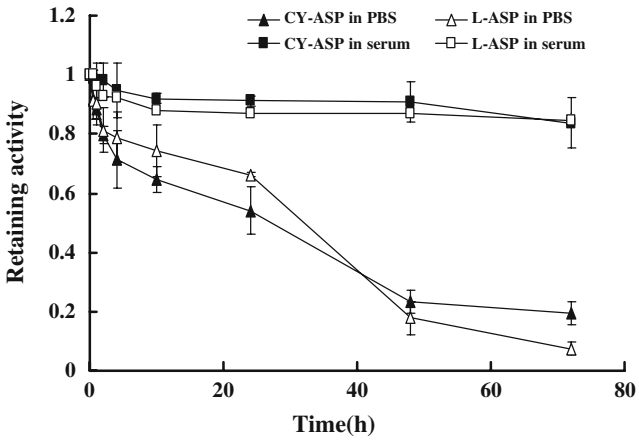

Fig. 7 Retention of L-ASNase activity in the presence of 20% DMSO in PBS and blood serum at 37 °C. The relative activity equaled to the ratio of every activity value to the highest one in the same curve

1 mg/ml was introduced to keep enough therapeutical efficacy and labeling ratio.

Stability of cypate–ASNase

To assess the stability of cypate–ASNase conjugation, the labeled complex and pure L-ASNase performed the parallel experiments and enzyme activity were measured. To obtain the retaining activities of the samples in different solutions, the initial enzyme activities for all the samples were designated as 1. As shown in Fig. 7, the enzyme activity of the labeled L-ASNase had similar behavior with that of native enzyme in same solution. However, significant difference existed in different mediums. The enzyme activity for both the labeled and native enzymes displayed very stable characteristics in serum. But a great decrease occurred in the PBS solution. It is conceived that some composition in serum such as albumin played an important role to protect enzyme activity. The stability of labeled enzyme in serum allowed us to perform in vivo measurement for a long period, which is suitable for in vivo monitoring of drug dynamic behavior.

NIR imaging of dynamics of labeled protein drug in mouse model

NIR fluorescence imaging system was utilized to trace the route of cypate-labeled L-ASNase in Kunmin mice. The optimal conjugation of cypate-labeled enzyme was administrated through tail vein into the blood stream of mice. The typical images for mouse no. 1 were displayed in Fig. 8. The cypate-ASNase complex was first accumulated in liver in the first 18 min and then sequestered in duodenum and intestines. The back and forth moving of cypate-labeled L-ASNase in duodenum and intestines was observed during the experiment. Two hours after drug injection, the fluorescence signal in liver gradually declined while the signal in intestines boosted up. The dye-labeled drugs were cleared from liver at about 130 min, but still observed in intestines 23 h after administration. These images revealed the clearance route of cypate-labeled L-ASNase, that is, from liver to intestines. The dynamic behaviors of cypate-labeled L-ASNase were similar in the group of five mice.

The above primary result demonstrated that NIR fluorescence probes incorporating with the NIR imaging system offered the unique capability of in vivo non-invasive tracing

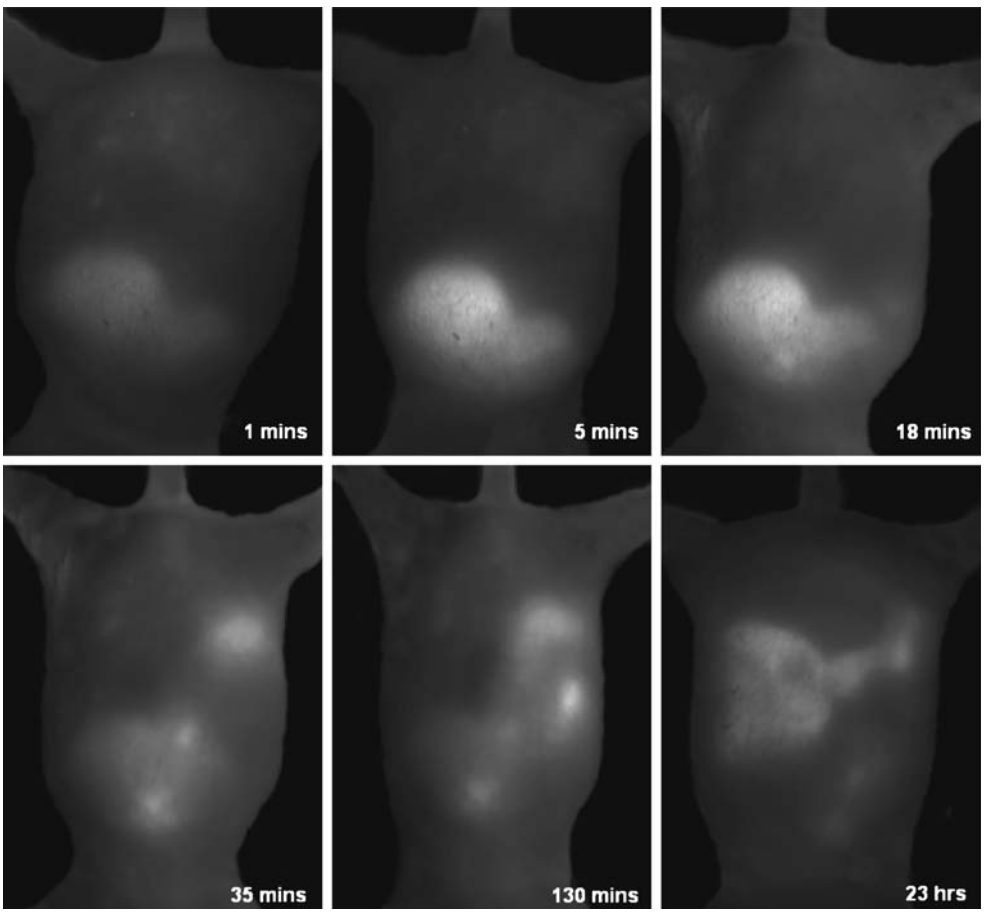

Fig. 8 Fluorescence imaging recorded at 1, 5, 18, 35, 130 min and 23 h after IV administration of cypate labeled L-ASNase

the dynamic signal in biological matrix. It exhibited great perspective in the pre-clinical application for novel drug development (drug screening, pharmacokinetic study, design of target carrier) and evaluation of therapeutic outcome. Compared to other imaging system for small animal experiments, such as MRI, SPECT and PET, NIR optical imaging provides a completely non-invasive monitoring modality with higher temporal resolution and relative low cost, although it has the limitation on the spatial resolution. The combination of different imaging modalities would greatly improve the imaging quality, and thus, to facilitate the application of imaging techniques in life science.

L-ASNase was used here just as a protein drug model for the in vivo investigation of its dynamic behavior in animal subject. In fact, the fluorescence labeling technique combining with the NIR imaging system could be easily translated to monitor the bio-distribution of other proteins in animal subjects. As the functional group in proteins for fluorescence labeling is the primary amino group provided by the ε-amino group of lysine or the N-terminus, most of the proteins contain such amino groups, and thus, could be conjugated to carboxy groups from NIR dye cypate. In the process of labeling different proteins to cypate, the optimal conditions for L-ASNase labeling could be followed and adjusted by changing the feed ratio of all the reaction components based on the number of functional lysines in proteins. In a word, the reported fluorescence labeling optimal conditions could be easily translated to other proteins, which significantly broaden the application of NIR technique in in vivo tracing different kinds of biomolecule in animal model. Furthermore, the NIR imaging system could be extended to monitor various optical signals by adjusting the excitation and emission filters.

Conclusion

In this study, the synthetic condition for cypate-ASNase conjugation was optimized to get better labeling efficacy. D/P ratios and enzyme activity of labeled protein were used as metrics to evaluate the labeling properties. pH condition, temperature, protein concentration and reaction duration were intensively investigated. Among these parameters, concentrations of L-ASNase, pH values and temperature played the important role in adjusting labeling efficiency. Reaction duration had less effect on the labeling efficiency. Moreover, the cypate-ASNase conjugate kept the enzymatic activity longer in the present of blood serum. The in vivo tracing of L-ASNase in animal model indicated that L-ASNase firstly accumulated in liver and subsequently transported to intestine 2 h after administration. The results obtained from NIR imaging demonstrated that NIR technique is a practical and reliable monitoring modality for in vivo tracing of drug concentration and distribution in small animal models. The procedures of this labeling method can be applied to most of the protein drugs. Therefore, the in vivo near infrared technique, including the labeling probe and monitoring tool, will have widespread application in protein drug development, which should significantly expedite drug screening and pharmacokinetic study.

Acknowledgment This work was partly supported by China National Natural Science Foundation (Grant No: 30371362, 30672015, 30700779) and the support from the Ministry of Education of China for returning people from oversea.

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