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International Journal of Pharmaceutics 337 (2007) 357–360
INTERNATIONAL JOURNAL OF PHARMACEUTICS
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Note

Effect of fiber length of carbon nanotubes on the absorption of erythropoietin from rat small intestine

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Received 28 April 2006; received in revised form 25 October 2006; accepted 30 December 2006
Available online 7 January 2007

Abstract

Erythropoietin (EPO) loaded carbon nanotubes (CNTs) with surfactant as an absorption enhancer were prepared for the oral delivery of EPO using two types of CNTs, long and short fiber length CNTs, and the effect of CNT fiber length on the absorption efficiency of EPO was studied. After Labrasol, PEG-8 caprylic/capric glycerides, as absorption enhancer was adsorbed into long fiber CNTs of which mean fiber length was 20–80 μm, as a carrier, EPO and casein as protease inhibitor and Explotab (sodium starch glycolate) as a disintegrating agent, were mixed. The resulting solid preparation was administered into the rat jejunum and serum EPO levels were measured by ELISA. The dose of EPO, CNTs, casein and Explotab were 100 IU/kg, 5 mg/kg, 25 mg/kg and 2.5 mg/kg, respectively. Serum EPO level reached to C_{max}, 69.0 ± 3.9 mIU/ml, at 3.5 ± 0.1 h and AUC was 175.7 ± 13.8 mIU h/ml. These values were approximately half of that obtained with short fiber length CNTs of which C_{max} was 143.1 ± 15.2 mIU/ml and AUC was 256.3 ± 9.7 mIU h/ml. When amphoteric surfactant, Lipomin LA, sodium β-alkylaminopropionic acid, was used to accelerate the disaggregation of long fiber length CNTs, C_{max} was 36.0 ± 4.9 and AUC was 96.9 ± 11.9, which showed less bioavailability (BA) of EPO. These results suggest that the short fiber length CNTs deliver more both EPO and absorption enhancer to the absorptive cells of the rat small intestine and the aggregation of CNTs is not the critical factor for the oral delivery of EPO.

Keywords: Carbon nanotubes (CNTs); EPO; Fiber length; Aggregation; Small intestinal absorption; Rats

1. Introduction

Recent development of nanotechnology has produced several new materials like carbon nanotubes, carbon nanohorns (CNT) and fullerene. The functions of these new materials are studied from the standpoint of semiconductor by Iijima (2002) and its various applications have been challenged (Baughman et al., 2002). CNTs are finite carbon structure consisting of needle-like tubes. These are produced using an arc-discharge evaporation method similar to that used for fullerene synthesis. The needles grow at the negative end of the electrode used for the arc discharge (Iijima, 2002). CNTs can be broadly classified into two categories: multi-walled nanotubes (MWNTs) and single-walled nanotubes (SWNTs). MWNTs comprises of carbon sheets co-axially arranged in a cylindrical shape. The number of coaxial tubes range from 2 to 50 and their diameter ranges between 1.4 and 100 nm (Iijima, 2002; Ding et al., 2001). Unlike MWNTs, SWNTs are formed in the gas phase (Iijima and Ichihashi, 1993). SWNTs show a diameter range from 0.4 to 3 nm (Ding et al., 2001). Invariably, both CNTs are capped at the ends. However, opening of CNTs have been reported and has been shown to be capable of obtaining capillarity-induced filling (Ajayan and Iijima, 1993; Tsang et al., 1993; Ajayan et al., 1993). In our previous study, short fiber length SWNTs, 2–3 μm, were used for the oral delivery of protein drug where erythropoietin (EPO) was used as a model protein. As the main utility of CNTs is an additive to iron products to increase the flexibility, longer fiber length CNTs are required. In this study, the effect of the fiber length of CNTs on the absorption of EPO has been studied in comparison with short fiber length CNTs.

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doi:10.1016/j.ijpharm.2006.12.042

2. Materials and methods

2.1. Materials

A commercially available erythropoietin injection (ESPO® 24,000 IU/0.5 ml) manufactured by Kirin Breweries (Japan) and marketed by Sankyo Corporation (Tokyo, Japan) was used. EPO ELISA kit was purchased from Roche Diagnostics GmbH (Germany). PEG-8 caprylic/capric glycerides (Labrasol®) (Gattefösse, Lyon, France) was a gift from Chugai Boeki Co., Ltd. (Tokyo, Japan). Casein was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Explotab®, sodium starch glycolate, (Penwest, USA) was obtained from Kimura Sangyo Co., Ltd. (Tokyo, Japan). Lipomin LA, sodium β-alkylaminopropionic acid, was obtained from Lion Co., Ltd. (Tokyo, Japan). All other materials used were of reagent grade and were used as received. Male Wistar rats used in the present study were obtained from Nippon SLC (Hamamatsu, Japan) and standard solid meal of commercial food (LabDiet®) was purchased from Nippon Nousan (Yokohama, Japan).

2.2. Preparation of CNTs containing EPO

CNTs, 10 mg, and Labrasol or Lipomin LA, 100 mg, were weighed into a bottle and mixed well. To the resultant mixture, Explotab, 5 mg, was added and mixed well using a micro-spatula so as to bring uniform distribution of the contents. EPO solution, 4.2 μl, was added to this mixture and finally, casein, 50 mg, was added and mixed well.

2.3. Absorption studies of EPO following administration of CNTs containing EPO to rat small intestine

Absorption studies were carried out with male Wistar rats (380–400 g body weight). Rats were fasted prior to 12–16 h with access to water *ad libitum*. The rats were anaesthetized by an intraperitoneal injection of sodium pentobarbital, 50 mg/kg. The hairs on the abdominal region were shaved and a 3 cm midline incision was made. The jejunum was exposed and CNTs containing EPO equivalent to an EPO dose of 100 IU/kg, was administered. The incision was sutured. The body temperature of the rats was maintained at 37 °C by heating with a lamp over the animals. Blood samples, 0.35 ml, were collected from the right jugular vein at 1, 2, 3, 4, 5 and 6 h after administration. In order to determine the bioavailability, (BA), EPO solution was injected into the left jugular vein at a dose of 50 IU/kg. Blood samples were collected from the right jugular vein at 2, 20, 40, 60, 120, 180, 360 and 480 min after administration. A blank blood sample was collected prior to the administration of the formulations. The animals were kept anesthetized throughout the experiment. Serum samples were obtained by centrifugation at 12,000 rpm for 15 min at 8 °C using Kubota 1700 centrifuge (Kubota, Tokyo, Japan) and stored at −80 °C until analysis. Experiment on animals was carried out in accordance with the Guidelines for Animal Experimentation in Kyoto Pharmaceutical University.

2.4. Serum EPO analysis by ELISA

The serum EPO level was determined by an ELISA method. Twenty microliters of the serum sample was used for analysis. The method involved the use of a standard EPO ELISA kit (Roche Diagnostics GmbH, Germany). The kit was slightly modified in the case of calibration standard samples wherein the use of ESPO at the concentration range as mentioned in the standard assay kit was used. This was done in order to overcome any difference between the supplied standard and the EPO used in the absorption studies. Accuracy of the standard concentrations was compared with the standard concentration supplied along with the kit. Accuracy level was found to be greater than 95%. All other reagents and procedure were used/carried out as mentioned in the supply manual. The ELISA plate was placed on a plate-shaker at 300 rpm/3 h/25 °C, (Titramax 101, Heidolph Instruments, Germany) and the ELISA plate was washed using a plate washer, Dia-washer II (Dia-Iatron Co., Ltd., USA). Finally, absorbance was measured at 450 nm using a micro-plate reader (MTP-300 microplate reader, Corona Electric, Japan).

2.5. Pharmacokinetic analysis

Pharmacokinetic parameters were determined from the serum EPO concentrations *versus* time data by a non-compartmental pharmacokinetic analysis method using WinHARMONY software developed by us (Yoshikawa et al., 1998). The maximum drug concentration (C_{max}) and the time to reach maximum concentration (T_{max}) were determined from the authentic serum concentration-time data. The area under the plasma drug concentration *versus* time curve (AUC) after intrajejunal administration was calculated using the linear trapezoidal rule up to the last measured drug concentration and BA was calculated by using the following equation.

$$\%\mathrm{BA} = \left(\frac{\mathrm{AUC_{jejunum}}}{\mathrm{AUC_{i.v.}}}\right)\left(\frac{\mathrm{dose_{i.v.}}}{\mathrm{dose_{jejunum}}}\right)100 \quad (1)$$

2.6. Statistics

All values are expressed as their mean ± standard error (S.E.). All values are expressed as their mean ± S.E. Statistical differences were assumed to be reproducible when $p < 0.05$ (Student's unpaired *t*-test).

3. Results and discussion

Two types of CNTs were used in this study. As shown in Fig. 1 which shows the SEM characteristics of CNT, short fiber length CNTs (Fig. 1a) has the mean fiber length of submicron to several micron and long one has 20–80 μm mean fiber length (Fig. 1b), respectively. Though the fiber length of CNTs differs between the two CNTs, the adsorption efficiency of CNTs against Labrasol was the same level.

To study the absorption enhancing effect of CNT system, *in vivo* absorption experiments were performed in rats and the results are shown in Fig. 2. After administration,

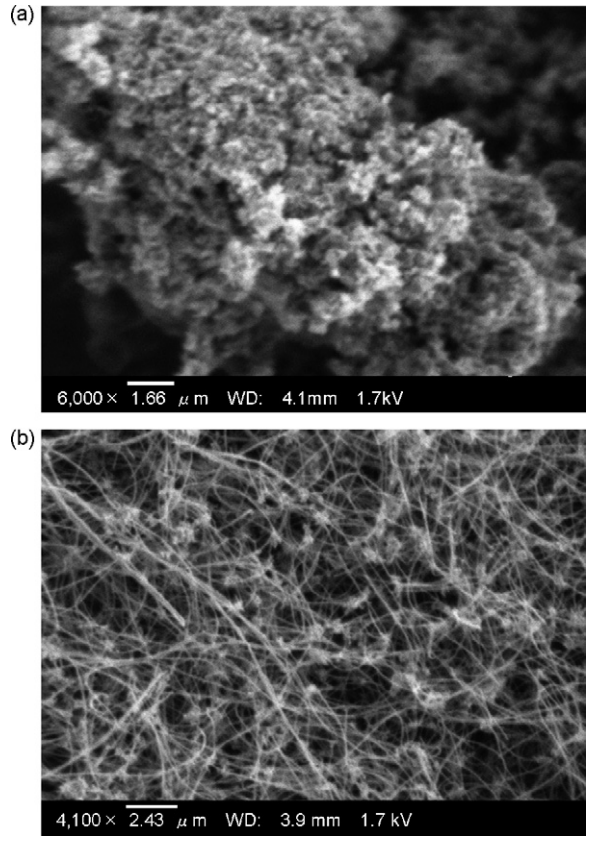

Fig. 1. SEM of CNTs used in this study. (a) Short fiber length CNTs, (b) long fiber length CNTs.

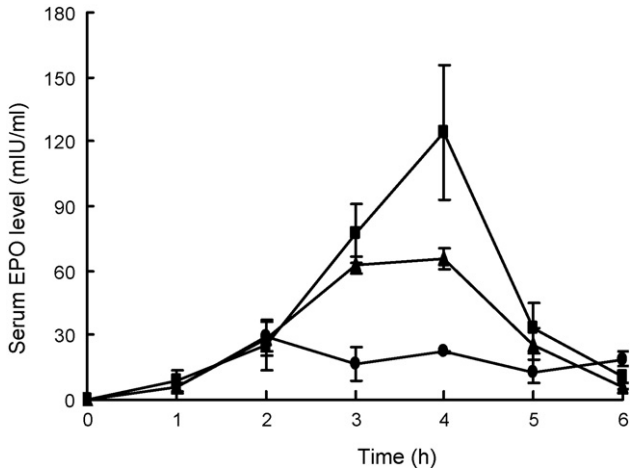

Fig. 2. Serum EPO concentration-time profiles after intra-jejunum administration of CNT preparations to rats, 100 IU/kg, (●) long fiber length CNTs with Lipomin LA, (△) long fiber length CNTs with Labrasol, (□) short fiber length CNTs with Labrasol. Each point shows the mean ± S.E. of four rats.

serum EPO level gradually increased and reached to its maximum level, C_{max}, 69.0 ± 3.9 mIU/ml, at 3.5 ± 0.1 h. Thereafter, serum EPO level decreased and reached to the pre-dose level at 6 h. Table 1 shows the results of pharmacokinetic analysis. The AUC was 175.7 ± 13.8 mIU h/ml. These values were approximately the half of that obtained with short fiber length CNT system of which C_{max} was 143.1 ± 15.2 mIU/ml and AUC was 256.3 ± 9.7 mIU h/ml. When amphoteric surfactant, Lipomin LA, was used to decrease the aggregation of long fiber length CNTs, C_{max} was 36.0 ± 4.9 mIU/ml and AUC was 96.9 ± 11.9 mIU h/ml. There was not a significant difference on T_{max} between the three CNT systems, 3.6 ± 0.3 h for short fiber length CNTs, 3.5 ± 0.1 h for long fiber length CNTs with Labrasol and 3.7 ± 0.5 h for long fiber length CNTs with Lipomin. However, C_{max} of long fiber length CNT system was about half of that obtained from short fiber length CNT system. By comparing the AUCs of CNT systems with that obtained after i.v. injection of EPO to rats, BAs were obtained. The BAs of long fiber length CNT system with Labrasol and Lipomin LA were 7.8% and 4.3%, respectively. These values were considerably lower that that of short fiber length CNT system with Labrasol.

CNTs are new materials and its application has been studied in many fields where the disaggregation of CNTs to each fiber has been found out to be the most important problem of CNTs. To smoothen the aggregation of CNTs and increase the solubility of CNTs, several methods like derivatization (Michelson et al., 1998), addition of solubilizing agents (Sun et al., 2001) were proposed. O'Connell et al. reported that CNTs were solubilized with the mixture of polyvinyl pyrrolidone (PVP) and surfactant, triton X-100 (O'Connell et al., 2001, 2002). According to their studies, amphoteric surfactant were more efficient than ionic surfactant to accelerate the solubility of CNTs. Therefore, Lipomin LA, a representative amphoteric surfactant, was used in this study and the effect of Lipomin LA on the intestinal absorption of EPO from CNT system was studied. By means of Lipomin LA-CNT system, the dispersion of CNTs was increased though the absorption enhancing effect was lower than CNT-Labrasol system. Therefore, the disaggregation of CNTs is not the critical factor for the absorption of EPO from CNT system. On the other hand, there was a great difference on the absorption enhancing effect of CNT system between long fiber length CNTs and short fiber length CNTs. The length of the used short fiber CNTs is approximately submicron to several microns and 20–80 μm for the long fiber CNTs have the mean diameter of 20–80 nm. Therefore, the short fiber length CNTs are physically easier to access to the enterocytes that locate on the intestinal

Table 1
Pharmacokinetic parameters of EPO after administration of CNT preparations to rats jejunum

Type of CNT (5 mg/kg)	Surfactant (50 mg/kg)	C_{max} (μg/ml)	T_{max} (h)	AUC_{0-6} (mIU h/ml)	BA (%)
Short fiber length	Labrasol	143.1 ± 15.2	3.6 ± 0.3	256.3 ± 9.7	11.5
Long fiber length	Labrasol	69.0 ± 3.9	3.5 ± 0.1	175.7 ± 13.8	7.8
Long fiber length	Lipomin LA	36.0 ± 4.9	3.7 ± 0.5	96.9 ± 11.9	4.3

AUC_{0-6}: area under the concentration–time curve after the administration from 0 to 6 h. BA: bioavailability (%) = $(AUC_{jejunum}/AUC_{i.v.}) \times (dose_{i.v.}/dose_{jejunum}) \times 100$ each value represents the mean ± S.E. (n = 3–4).

microvilli than longer fiber length CNTs. Our results suggest that short fiber length CNTs deliver more both EPO and absorption enhancer to the enterocytes than longer fiber length CNTs and the contact time of them to enterocytes were increased.

Many scientists including us have been studying the oral delivery of peptide/protein drugs like insulin using protease inhibitors (Paolo et al., 2000; Lowman et al., 1999), absorption enhancers (Eaimtrakarn et al., 2002; Shen and Mitragotri, 2002; Patel et al., 1991), bioadhesive polymer modified liposomes (Morishita et al., 1998), Chitosan capsule (Ito et al., 2005), water-in-oil-in-water double emulsions (Iijima, 2002; Baughman et al., 2002; Ding et al., 2001). However, oral insulin DDS has not been developed yet, because of low oral BA. To increase the BA of EPO, novel DDS is needed. Under these background, CNTs were used for the oral delivery of EPO and CNTs have been found out to be a good carrier for oral delivery of protein drugs. However, CNTs are extremely new material. Therefore, safety problem must be solved to use CNTs as pharmaceutical additives, because several studies suggested the lung toxicity of CNTs (Lam et al., 2004; Warheit et al., 2004).

In conclusion, the effect of CNT fiber length on the absorption of EPO from rat small intestine has been studied. The absorption enhancing effect of longer fiber length CNTs using Labrasol as an absorption enhancer was lower than that of short fiber CNTs. The disaggregation of CNTs by an amphoteric surfactant was shown no to be the critical factor for the oral absorption efficiency of EPO.

Acknowledgments

This study was supported by a “Collaboration with Bio-Venture Companies” Project for Private Universities: matching fund subsidy from MEXT (Ministry of Education, Culture, Sports, Science and Technology, MEXT), 1999–2006 of Japan. This study was also supported by a Grant-in-Aid for Scientific Research (B) provided by the Ministry of Education, Science and Culture of Japan.

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