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Research on Thymopentin Loaded Oral *N*-Trimethyl Chitosan Nanoparticles

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Peptides, although high efficacy and specificity in their physiological function, usually have low therapeutical activities due to their poor bioavailability when administrated orally. Nanoparticles have been regarded as a useful vector for targeted drug delivery system because they can protect drug from being degraded quickly and pass the gastrointestinal barriers. Here we described a novel oral N-trimethyl chitosan nanoparticles formulation containing thymopentin (Tp5-TMC-NP). N-trimethyl chitosan (TMC) was synthesized and then used to prepare Tp5-TMC-NP by ionotropic gelation. A three-factor, five-level CCD (Central Composite Design) design was used in the optimization procedure, with HPLC as the analyzing method. The resulting Tp5-TMC-NP had a regular spherical surface and a narrow particle size range with a mean diameter of 110.6 nm. The average entrapment efficiency was 78.8%. The lyophilized Tp5-TMC-NP formulation was stable in 4°C or -20°C after storage of 3 months without obvious changes in morphology, particle size, pH and entrapment ratio. The results of the flow cytometer determination showed that the ratio of $CD4^+/CD8^+$ of Wistar female rat givenTp5-TMC-NP (ig) was 2.59 time that of the group given Tp5 (ig).

Key words: Thymopentin (Tp5), Oral drug delivery system, Nanoparticle, *N*-Trimethyl chitosan (TMC)

INTRODUCTION

Most peptide preparations are administered parenterally up to date. Non-parenteral routes of administration are appealing more interests today. Among them oral dosage form is the focus for its convenience and high compliance. However, administrating polypeptide drug orally has a bioavailability of only 0.1%-2% or even lower, the barriers for oral polypeptides administration are tight epithelial barriers, gastric acid and enzymes, which defined the biomembrane and enzyme barrier. Tight junction is the major obstacles. Liposome, considered as an artificial biomembrane, is apt to cell fusion and pinocytosis, so relatively easy to deliver drugs to cells, but it has poor stability (Sahli *et al.*, 1998 and Huguette *et al.*, 2000). Nanoparticle is an immunogenicity free, stable and less toxic drug delivery system that easy to prepare and lyophilized (Chaco'n *et al.*, 1999). Evidence reveals that nanoparticles between 40~200 nm may be absorbed by the way of either paracellular uptaking or endocytosis (Mathiowitz *et al.*, 1997).

Chitosan (CT) can trigger the opening of the tight junctions due to an interaction of the positively charged amino group with the negatively charged sites on the cell surfaces and tight junctions (Artursson *et al.*, 1994 and Ranaldi *et al.*, 2002) thereby facilitating the paracellular transport of hydrophilic compounds. But in solutions at pH values above 6.5, the chitosan will lose its charge and precipitate from solution. This property implies that chitosan can be effective as an absorption enhancer only in a limited area of the intestinal lumen where the pH values are close to its p*K*. In this regard, possibilities for the potential use of chitosan as an absorption enhancer in the basic environment of the large intestine,colon and rectum, are limited. However, *N*-trimethyl chitosan chloride, a chitosan derivative could overcome this problem, reduce the transepithelial electric resistance in a reversible way to open the tight junction and allow for paracellular transport of hydrophilic macromolecules, which make it an excellent

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material in large intestine and colon drug delivery systems. Studies on the toxicity of TMC on Caco-2 cells reveal that TMC-polymers are non-toxic to intestinal epithelia (Maya *et al.*, 1999).

Protein or polypeptide drugs, loaded in TMC nanoparticles, will benefit from absorption enhancement and physiological enzymolysis avoidance (Thanou *et al.*, 2000). In 1979 Glodstein synthesized a 32^{th}-36^{th} sequence (Arg-Lys-Asp-Val-Tyr) pentapeptide of the thymopoietin and named it thymopentin (Tp5), which is considered the active site and remained the effective bioactivity. Tp5 is a cellular immunomodulating drug and can induce T cell differentiation and promote T lymphocyte subpopulation development and activation. Tp5 is not stable *in vivo* and is easy to be degraded taken orally, so formulation of oral Tp5 nanoparticles is of great scientific and practical significance.

In the present study, Tp5-TMC-NP was prepared with N-trimethyl chitosan as the vector and thymopentin, the immunomodulator and immunoenhancer, as the candidate.

MATERIALS AND METHODS

Materials

Chitosan (CT, DD90%, viscosity<100cps, MW 56k) was purchased from Boao Bio.Co. (P. R. C.). Tymopentin was purchased from Yuanji Phar. Co. (PRC). Cyclophosphamide (CTX) was obtained from Hualian Phar. Co. (P. R. C.). Hemolysin, FACSTM Lysing Solution was purchased from Becton-Dickinson (U.S.A.). ANTI RAT CD3: FITC/CD4 RPE-DUAL REAGENT and ANTI RAT CD3: FITC/CD8 RPE-DUAL REAGENT were purchased from Serotec Ltd. (U.S.A.). All the other chemicals and reagents used were of the analytical grade obtained commercially.

Synthesis of TMC

The method of two-step synthesis was used in order to get TMC according to Sieval's report (Sieval *et al.*,1998). The degree of substitution (D.S.) was calculated as the following:

$$D.S. = \frac{\int \text{peak at 3.3 ppm}}{\int \text{peak from 4.7 ppm to 5.5 ppm}/9}$$

And the yield of the TMC was calculated as:

yields% = chitosan addition/TMC obtained×100%

Tp5-TMC-NP Preparation

We prepared the Tp5-TMC-NP by the ionotropic gelation, so as to provide the peptide a rather mild condition and get a uniform and adjustable sized particle. Algin solution was dropped to the TMC solution with a sufficient amount of Tp5 by a syringe with magnetic stirring and keeps stirring for 10 minutes when the positive charged algin would be adsorbed to TMC and resulted in precipitation, thus TMC-NP were formed.

Size and the entrapment efficiency

The size of the nanoparticle is thought to be critical for *in vivo* delivery because particle size influences not only the biodistribution but also the efficiency of cellular uptake through macrophages. It is observed that particles with the size of 40-200 nm can be absobed by both macrophage uptake and paracellular route. Entrapment efficiency is a conventional index for nanoparticle evaluation. Therefore entrapment efficiency and particle size were chosen as responses of CCD. Entrapment efficiency was studied by HPLC (CTO-10AVP Shimadzu, JP) with a mobile phase of PBS (0.1 mol·L^{-1}, pH 7.0)-acetonitrile (94-6) and a wave length 275 nm. Tp5-TMC-NP colloidal solution was freeze-centrifugalized under 4°C, 15000 r·min^{-1} for 2 h and the supernatant examined by HPLC. The entrapment efficiency (EE%) was calculated according to the following equation:

$$EE\% = [(X_t - X_f)/X_t] \times 100\%$$

Where X_t is the total Tp5 amount in the colloidal solution, X_f is the amount of Tp5 in the supernatant. The particle size was examined by Nano Sz900 laser nanogranulometer (Malvern, Britain). The colloidal solution was added to a certain amount of water and stirred, then pumped to sample cell and examined.

Central composite design

Central composite design (CCD), also called Response Surface Methodology (RSM), is a rapid technique used to empirically derive a functional relationship between an empirically response and a set of input variables. Furthermore, it may determine the optimum level of experimental factors required for a given response. Values of each factor can be set during the experiment as input variables, which would affect the response variable as is measured from the experiment. CCD reduces the number of experimental runs that are necessary to establish a mathematical trend in the experimental design region (Xun *et al.*, 2004).

According to the single-factor experiments, 3 major factors were chosen for the further optimizing experiments, which were the *N*-trimethyl chitosan (TMC) concentration (X_1), the Sodium Alginate concentration (X_2) and the amount of Tp5 addition (X_3). A three-factor, five-level central composite design was employed. Each response was derived as desirability between 0 and 1, so there would be two desirabilities as is designed. In order to get a total evaluation of the particle property we derived an overall desirability by the geometric mean of the two desirabilities.

Preparation of lyophilized Tp5-TMC-NP

Nanoparticle in colloidal solution is not stable enough for clinical use, so we have the Tp5-TMC-NP lyophilized. To get an ideal lyophilized preparation several conventional supporting agents as glucose, lactose and mannitol were investigated, the appearance, color and redispersibility were chosen as evaluation indexes.The *in vitro* Stability of the lyophilized Tp5-TMC-NP was also studied.

Tp5-TMC-NP's influence on the value of $CD4^+$/ $CD8^+$ by the flow cytometer

Tp5 has a specific effect in inducing and promoting differentiation, maturation and activating of T-lymphocytes and the subpopulation. It is capable of regulating and normalizing the ratio of $CD4^+/CD8^+$ of the T-lymphocytes. However, *in vivo* studies shows that Tp5 has a very short half life which made it difficult to directly determine the blood drug level. According to its biological property as an immunoregulant, flow cytometer was employed to examining the changes of immune factors after given Tp5.

Wistar female rats were given Cyclophosphamide (CTX) by peritoneal injection, according to body weight, to get an immune suppression 3 days before Tp5-TMC-NP intragastric administration. Wistar rats were divided into 6 groups randomly. Each group, composed of 5 rats, were given Tp5 (IV), Tp5-TMC-NP (ig), Tp5-CT-NP (ig), Tp5+ TMC (ig), Tp5 (ig) and saline (ig) respectively, with the dosage calculated according to surface area, once a day for 7 days. On the 8^{th} day, 2 mL of blood samples were obtained from fossa orbitalis for the subsequent determination. 10 μl of ANTI RAT CD3: FITC/CD4:RPE-DUAL REAGENT or ANTI RAT CD3: FITC/CD8 RPE-DUAL REAGENT were added to 100 μL of whole blood samples and swirled to mix them fully, then incubated for 30 minutes avoid from light. Then 1:10 diluted hemolysin were added and centrifugated at 500~800 rmp then washed with PBS for 3 times and centrifugated again for 5 minutes followed by determination by Flow cytometer (FACS scan Becton, Dickinson, U.S.A.). Data from the experiments were analyzed by the statistical package Cellquest 2.0.

Table I. Determination of experimental values for the CCD with a specified axial apacing

	Axial star point (lower)	Factor point (lower)	Central point	Factor point (upper)	Axial star point (upper)
X_1	0.5	0.92	1.5	2.08	2.5
X_2	0.5	0.92	1.5	2.08	2.5
X_3	2	2.42	4	5.58	6

Table II. Observed responses and derived desirability for the CCD design

No.	X_1	X_2	X_3	D(nm)	EE(%)	D_1	D_2	OD
1	0.92	0.92	2.42	108.2	65.2	0.9119	0.304	0.5265
2	0.92	0.92	5.58	106.9	65.3	0.9159	0.306	0.5294
3	0.92	2.08	2.42	158.9	67.8	0.7534	0.356	0.5179
4	0.92	2.08	5.58	160.3	73.7	0.7491	0.474	0.5959
5	2.08	0.92	2.42	213.6	76.3	0.5825	0.526	0.5535
6	2.08	0.92	5.58	216.4	80.6	0.5738	0.612	0.5926
7	2.08	2.08	2.42	210.7	71.4	0.5916	0.428	0.5032
8	2.08	2.08	5.58	218.9	83.1	0.5659	0.662	0.6121
9	0.5	1.5	4	102.4	58.3	0.93	0.166	0.3929
10	2.5	1.5	4	388	69.4	0.0375	0.388	0.1206
11	1.5	0.5	4	117.9	73.2	0.8816	0.464	0.6396
12	1.5	2.5	4	104.8	79.8	0.9225	0.596	0.7415
13	1.5	1.5	2	110.5	76.1	0.9047	0.522	0.6872
14	1.5	1.5	6	132.8	84.3	0.835	0.686	0.7568
15	1.5	1.5	4	109.7	80.2	0.9072	0.604	0.7402

X_1: the concentration of TMC (mg/ml)
X_2: the concentration of algin (mg/ml)
X_3: the amount of Tp5 addition (mg)
D: Tp5-TMC-NP size (nm)
EE: entrapment efficiency
D_1: desirability of Tp5-TMC-NP size
D_2: desirability of entrapment efficiency
OD: overall desirability.

RESULTS AND DISCUSSION

Results of *N*-trimethyl chitosan synthesis

IR spectrum of the TMC obtained after the two-step reaction clearly shows two strong sharp peaks at 1476 cm^{-1} and 1376 cm^{-1} which indicates the existence of methyl, different from that of the 1450~20 cm^{-1} peak in conventional chitosan's spectrum. The ^{1}H-NMR spectrum of TMC shows a sharp peak at 3.3 ppm assigned to $N(CH_3)_3^+$. Yield of the reaction was 98% and the degree of substitution was 64.39%. Thus the TMC synthesized suited the subsequent Tp5-TMC-NP preparation.

Optimization of the Tp5-TMC-NP formulation

The experimental run and the observed responses of the 15 formulation were given in Table I and Table II together with the desirability derived form the responses. The constant, the regression coefficient for each response variable were listed in Table III.

According to the response surface derived, by STS-TISTICA, from the regression equations the predicted ranges or points of the independent variables were defined as was shown in Table IV.

Ranges of the factors in Table IV were overall evaluated and the optimum formulation was decided as concentration of TMC 1.5 $mg \cdot mL^{-1}$, concentration of algin 1.5 $mg \cdot mL^{-1}$ and the amount of Tp5 addition 5 mg.

Predicted responses of the optimum formulation derived from the regression equations in Table III were compared with the responses observed from the optimum preparation and the results were shown in Table V.

The optimal Tp5-TMC-NPs prepared had an average entrapment efficiency of 78.8%, and the Zeta potential was 41.2 mV. The transmission electron microscope (TEM) micrograph showed that the Tp5-TMC-NPs were uniform globules (Fig. 1) with a mean diameter of 110.6 nm.

Table III. Regression equations for the responses

$$D = 301.8456 - 278.594X_1 + 33.41339X_2 - 49.7129X_3 + 145.2670X_1^2 - 41.3702X_1X_2 + 0.534325X_1X_3 + 11.41699X_2^2 + 0.152395X_2X_3 + 6.184453X_3^2 + 0.634981X_1X_2X_3 \ (R = 0.95464)$$

$$EE = 13.30182 + 61.97745X_1 + 17.51427X_2 - 0.831965X_3 - 16.8232X_1^2 - 6.48433X_1X_2 + 0.799606X_1X_3 - 4.17324X_2^2 + 1.236096X_2X_3 - 0.154901X_3^2 + 0.376285X_1X_2X_3 \ (R = 0.99135)$$

$$OD = -0.425102 + 1.397261X_1 + 0.125192X_2 - 0.002114X_3 - 0.479540X_1^2 - 0.027973X_1X_2 + 0.011022X_1X_3 - 0.045740X_2^2 + 0.021635X_2X_3 - 0.003270X_3^2 - 0.001246X_1X_2X_3 \ (R = 0.95692)$$

D: Tp5-TMC-NP size (nm)
EE: entrapment efficiency
OD: overall desirability

Table IV. Predicted Optimum Ranges (Points) of the Independent Variables

Response Variable	Predicted Optimum Range (Point)		
	C_{TMC}	C_{Algin}	C_{Tp5}
Size	0.5-1.5	1.0-1.5	2.0-5.5
EE	1.5-2.0	1.0-2.0	4.0-6.0
OD	1.0-2.0	1.5-2.5	4.0-6.0

Stability study *in vitro* of the lyophilized Tp5-TMC-NP

According to our investigation we chose 3% mannitol as the supporting agents and three batches of the lypholized preparation were prepared. The lypholized Tp5-TMC-NP were uniform white puff powders with sufficient intensity and a smooth surface,and easy to disperse in water. The particle size and drug loading efficiency showed no difference before and after the lyophilization process. The lypholized Tp5-TMC-Np were stable at 4°C and -20°C for 3 months as shown in Table VI.

The result of influence on the value of $CD4^+/CD8^+$ by the flow cytometer

Given peritoneal injection of CTX for three days the rats

Table V. Comparison of the observed and predicted Values of the response variables of the nanoparticles prepared under the optimum condition

Response variables	Predicted Response	Observed Response	Bias (%)
D	107.3	110.6	-3.08
EE(%)	79.8	78.8	-1.25
OD	0.7384	0.7218	-2.25

Bias was calculated as (predicted value–observed value)/predicted value × 100%
D: Tp5-TMC-NP size (nm)
EE: entrapment efficiency
OD: overall desirability.

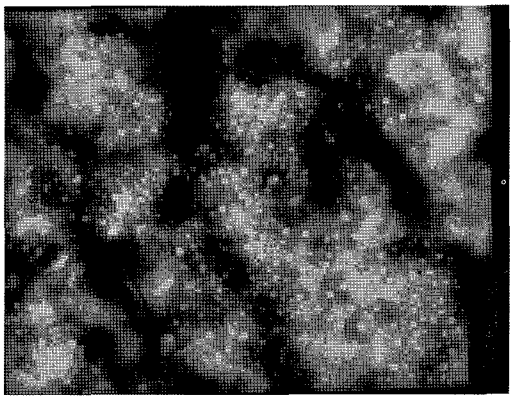

Fig. 1. The TEM micrograph of Tp5-TMC-NP

Table VI. The stability of lyophilized Tp5-TMC-NP

	Time (month)	EE (%)	pH	Size (nm)
	0	78.75	5.49	108.4
4°C	1	79.34	5.63	112.4
	2	77.68	5.84	118.1
	3	80.47	5.42	111.7
-20°C	1	76.65	5.66	112.8
	2	76.12	5.59	116.7
	3	78.04	5.62	117.5

Table VII. Result of checking the $CD4^+/CD8^+$ (n=5)

Group	Treatment	$CD4^+/CD8^+$	Dosage (mg/kg)	$(CD4^+/CD8^+)$/ Dosage
A	Tp5 (iv)	4.41	0.09	48.96
B	Tp5-TMC-NP(ig)	5.08	0.90	5.65
C	Tp5-CT-NP (ig)	2.06	0.90	2.29
D	Tp5+TMC (ig)	2.03	0.90	2.26
E	Tp5 (ig)	1.97	0.90	2.18
F	Saling (ig)	1.94	0.90	2.16

were physiologically weak which showed the formation of immunosuppression. Results obtained by the flow cytometer (Table VII) showed that the value of $CD4^+/CD8^+$ of group given Tp5-TMC-NP (ig) was 2.59 time that of the group given Tp5 (ig), but much smaller than that of group given Tp5 (iv).

CONCLUSIONS

The present study concerned itself with the preparation of Tp5-TMC-NP and focused on the optimization of the preparation and the enhancement of the oral effect. It was concluded that the optimal Tp5-TMC-NP we prepared, and the Tp5 using TMC-NP as its vector can increase the oral effect according to the value of $CD4^+/CD8^+$.

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