Self-Assembled Chitosan-g-Pluronic F-127 Copolymer (ChPC) Loaded with Polydatin Nanoparticles: Implication as Anti-Diabetic Therapy

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Received date: April 22, 2020; Accepted date: May 04, 2020; Published date: May 11, 2020


Abstract

Our aims to develop a safe and effective polymeric nano-template for assessing pharmaceutical potentialities in modulating the drug profile in the field of anti-diabetes research. We rationally aimed to design the Chitosan (Ch) grafting with PF as co-polymer (ChPC). The FT-IR, dynamic light scattering, SEM, TEM and % entrapment efficacy are commenced to examine the efficacy of the prepared nanoparticles in successful Polydatin (PD) delivery (PD-ChPC-NPs) having average particle size 263 ± 1.25 nm with PDI 0.162. The PD-ChPC-NP has a spherical in shape and can be loaded with high encapsulation efficiency (86.49 ± 0.29). It was observed that PD-ChPC-NP was stable at various temperatures and biocompatibility. In vivo animals studies have also clearly shown that compared with free PD, PD-CSNP has a significant anti-diabetic effect in diabetic rats. To conclude, the current research shows that PD-ChPC-NPs represent potentially safe nanocarriers that can be used for non-toxic and effective treatment of diabetes.

Keywords: Polydatin; Chitosan; Pluronic F-127; Nanoparticle; Cytotoxicity; Diabetes treatment

Introduction

Diabetes mellitus (DM) is a chronic and serious metabolic diseases. The approximated global forecasts will increase more than 600 million patient have diabetes in 2040 [1]. DM is characterized by high blood glucose level and hindrance comes across in the production of insulin. DM is associated with many complications consist of cardiovascular disorder, renal failure, liver toxicity, obesity and insulin dysfunction caused by loss of cell function [2]. Prevention of these complications arising in type 1 DM and further conversion to type 2 DM which is important to improve the quality of life [3]. Synthetic treatments of DM have some limitations due to expensive and produce adverse effect. In this regards, it is very important to prepare effectively protective hypoglycaemic agents from natural plants as a substitute for DM control. Polydatin (Pd), is also called as piceid (3,4’-5-trihydroxystilbene-3-β-d-glucoside). It is a glycoside of resveratrol and isolated from dried rhizome of Polygonum cuspidatum [4]. As previously reports of pharmacological analysis and clinical practices verified that PD has shown a therapeutic effect in the patient suffering from renal failure, cardiac disorder, diabetes and hypertension [5,6]. Unfortunately, due to poor water solubility, chemically unstable in alkaline aqueous solutions and low bioavailability, its clinical application is limited [7]. Therefore, several attempts have been made to overcome these deficiencies via drug delivery system (DDS), prolonged release kinetic, preventing degradation and improve the water solubility of loading drug [8]. To address these concerns, PD can be loaded within biodegradable polymers to improve its solubility, bioavailability and reducing the toxic profile.

Pluronic F-127 (PF) is non-ionic tri-block, and bio-degradable polymer has been endorsed by the US Food and Drug Administration (FDA), can be used as an excellent pharmaceutical excipient [9]. PF having high critical micelle concentration, dissociation of targeted DDS has occurred. This problem can be solved by coupling with other polymers. Herein, we have designed Chitosan (Ch) grafting with PF as co-polymer. Ch is a poly-cationic polymer and famous natural biodegradable, which confirm increase stability. Previous investigation of co-polymer grafting like stearic acid with PF loaded with doxorubicin nanoparticles [10], and it has been widely reported in the literature that poly (lactic acid) -PF for oral administration of insulin improves the targeted efficacy of drugs [11].
These studies inspired us to further develop a Ch grafting with PF copolymer (ChPC), followed by to develop a self-assembly nanoparticles loaded with PD known as Pd-ChPC-NPs or un-loaded refers as ChPC-NPs for comparative studies. Its positively charged surface can promote cell membrane penetration and fixation of mucous glycoproteins [12]. We have prepared and characterized the physicochemical properties and evaluated their safety and potential efficacy against DM by using in vitro study and in vivo on rodent.

Materials and Methods

Polydatin (PD), Chitosan (Ch) (300 kDa, 75 – 85 % deacetylated), Pluronic F127 and MES (2-(N-morpholino) ethanesulfonic acid) was obtained from (Sigma- Aldrich USA). Succinic anhydride, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and N-hydroxysuccinimide (NHS) (Aladdin co. Ltd, China). Cellulose membrane (molecular weight cut-off = 3000, MWCO) were obtained from (Sangon Biotech, Shanghai, China). All chemicals were analytically pure and obtained from standard commercial supplies for future studies.

Cell culture

The human colon adenocarcinoma cell lines (HT29) were cultured in Roswell Park Memorial Institute medium (RPMI-1640) supplemented with 10 % fetal bovine serum (FBS) at 37°C in humidified atmosphere air containing 5 % (v/v) CO2 mixture.

Animal host

Wistar rats (average body weight (BW) 100-130 gram) were used as the experimental host (Medical Research Institute, Hubei University of Science And Technology, program number: DFKY163953). The rats were housed in a climate-controlled room in a cage (light time 12 h to dark time 12 h cycle) and feed with standard rodent pellet ad libitum and normal water. Male rats were kept in a controlled humidity and temperature. Animals were housed in pathogen free lab

Chitosan-g-pluronic F-127 copolymer (ChPC)

ChPC was synthesized with different steps of chemical reaction as reported method with slightly modification[13] First, under a nitrogen atmosphere, PF was mixed with DMAP, TEA and succinic anhydride in 1,4-dioxane for carboxylation. The resultant was washed thrice with diethyl ether followed by lyophilisation. Second, lyophilized mono carboxylated PF conjugated with amino groups of Ch using NHS/EDC in MES buffer (pH 4-6) to formed ChPC. As shown in Figure 1, ChPC was synthesized by grafting with mono-carboxyl PF with Ch. The final product was introduced for dialysis bag in water and lyophilized.

Preparation of ChPC-NPs

ChPC-NPs were prepared by using ionic gelation method with slight medication [14]. Briefly, Ch solution (0.5 %, w/v) was synthesized by dissolving in glacial acetic acid solution (2 % v/v) with pH 4.8 containing Tween-80 (1 %) for constant stirring for 12 h. For ChPC-NPs preparation, the TPP solution was added drop-wise to ChPC solution and kept under stirring for 2 h at 900 rpm, thereby leading to formed ChPC-NPs were separated by centrifugation and washed.

Preparation of PD-loaded ChPC-NPs (Pd-ChPC-NPs)

For Pd-ChPC-NPs preparation, PD solution was added drop-wise to ChPC-NPs (1:3, w/w). The mixture was continuously stirred 30 minutes at 1000 rpm and sonicated for 10 min by using a probe sonicator to formed Pd-ChPC-NPs. The formed Pd-ChPC-NPs solution was centrifuged (12,000 rpm, 30 min) at cooling temperature (4°C). The final product was dispersed in water and lyophilized to obtain for further studies.

Characterization

Particles size, distribution and zeta potential: The average particles size, distribution and poly-dispersion index (PDI) of prepared PD-ChPC-NPs and CPC-NPs were evaluated by dynamic light scattering (DLS) analysis by using (Nano ZS Zetasizer, Malvern Instruments Corp, UK) at 25°C. The zeta potential was also calculated by using Zetasizer (Nano-ZS, Malvern Instruments Ltd, UK) All the measurements were carried out in triplicate.

Scanning Electron Microscopy (SEM): The morphology of the sample was detected by SEM (JEOL, JSM-6700F), and it was worked at an acceleration voltage of 20 kV. The Edwards S 150 sputter coater (Agar Scientific, Standsted, UK) was used to plate NPs with a thickness of approximately 150 Å.

Transmission Electron Microscopy (TEM): The sample was analysed for the morphological characterization of PD-ChPC-NPs using TEM (JEM 2100 LaB6, JEOL, and Japan). The diluted PD-ChPC-NPs was used and placed on a carbon-coated Cu grid (200-mesh), then stained with phosphotungstic acid (2 % PTA) solution, and desiccated at room temperature.

Estimation of percent entrapment efficiency (% EE): The amount of drug (PD) was determined using UV/VIS spectrophotometric method. The sample was centrifuged (12000 rpm for 30 min at 4°C) for isolated the free PD. The un-entrapped PD was detected at a λ= 305 nm. The percentage of PD entrapment efficiency (% EE) was calculated using the following equation:

\[ %\text{EE} = \frac{\text{weight of total PD amount} - \text{weight of un-entrapped PD}}{\text{weight of total PD amount}} \times 100 \]

In vitro release study

The in vitro release of PD from PD-ChPC-NPs and free-PD were attained in PBS, at pH 7.4 by using USP dissolution test apparatus II at 100 rpm [15]. Briefly, dissolution apparatus was filled with dissolution medium and immersed in a water bath at temperature 37 ± 0.5°C. The PD-ChPC-NPs and Free-PD were
placed in a different dialysis bag and immersed in the dissolution medium. 1 ml sample was withdrawn at a different time intervals and replenished with fresh medium to maintain the sink condition. The released PD concentration was recorded using a spectrophotometer (λ= 305 nm). All experiments were repeated triplicate.

Stability studies

To calculate stability study of PD-ChPC-NPs according to ICH guidelines to evaluate the effect of different temperature (4 ± 2 °C and 25 ± 5 °C) for 6 weeks [16]. The different characteristics of the samples were analysed, such as particle size, and polydispersity index. All measurement were carried in a triplicate (n=3).

% Cell viability of PD-ChPC-NPs

The cell viability profile of PD-ChPC-NPs and ChPC-NPs on HT29 cell lines were examined by using MTT study [17]. Cells were cultured in 96-well plate (5000 cells in each well) for 12h, and then treated with PD-ChPC-NPs, Ch-NPs, and free-PD (as a control) at 37°C for 24 h (5 % CO₂) at different concentration. The cells were washed with PBS, and 10 µL MTT solution (5 mg ml⁻¹ in PBS) was dispensed into each well for 4 h. Further, the culture solution was discarded from each well, and DMSO (150 µl in each well) was mixed to solubilize the purple formazan precipitate in the dark for at least 10 min. The absorbance of formulations was calculated using ELISA reader (Thermo Scientific, USA) at 570 nm.

In vivo study

Induction of diabetes in rats: The rats were fasted overnight and, induced diabetes by intraperitoneally (i.p.) administering of prepared streptozotocin (STZ, 50 mg/kg BW) solution in 0.1 M citrate buffer at pH 4.5 [18]. After 7 days of the administration, the blood glucose levels of rodents were monitored using a glucometer (Accu-Check, Roche, USA) until the rodents with fasting blood glucose (FBG) level ≥ 250 mg/dl were determined to have stable diabetes and ready for further analysis.

- Animals were allocated randomly in six groups (n=6)
- Control animals group – CG
- Diabetic animals group – DG
- Diabetic animals treated with PD (50 mg/kg BW) – D-PD oral administration for 28 days.
- Diabetic animals treated with PD-ChPC-NPs (50 mg/kg BW) – D-PD-ChPC-NPs oral administration for 28 days.
- Diabetic animals treated with ChPC-NPs (50 mg/kg BW) – D-ChPC-NPs oral administration for 28 days.
- Diabetic animals treated with metformin HCl (100 mg/kg BW) – D-MET oral administration for 28 days. To calculate the BW of each group from initial treatment to final treatment were recorded.

Biochemical examinations

FBG concentration was calculated by using a glucometer (Accu-Check, Roche, USA) in serum samples collected from the lateral tail vein once a week, and the maximum measurement volume is 600 mg / dL. Besides, the percentage (%) of blood glycosylated hemoglobin (Gly-Hb) was evaluated using a purchased kit (Bio-systems, Barcelona, Spain), and fasting blood insulin (FBI) levels were measured by ELISA detection kits (DRG, international, Germany).

After 28 days, at the end of experiments, before collecting serum samples in heparin test tubes, the rats were anesthetized with chloroform. The serum samples were isolated by centrifugation (3,000 rpm, at 4°C) for 10 min for further evaluation of the biochemical parameters. The lipid parameters such as serum cholesterol, triglycerides level [19], and liver serum biomarkers namely alkaline phosphatase (ALP), serum alanine transaminase (ALT), and aspartate aminotransferase (AST) were calculated by commercially available diagnostics kits [1].

Statistical analysis

Statistical analysis was calculated by single a analysis of variance (ANOVA) method using Graphpad prism software (Graph Pad Software Inc., CA, USA) and Turkey–Kramer. Significance levels were used P < 0.05, P < 0.01 and P < 0.001 for our analysis. Results have been indicated as mean ± standard deviation (SD) values (n =6).

Results and Discussion

Characterization of ChPC

Polymeric nanoparticles have been studied to encapsulated active drugs with different advantages to improve their solubility, cost-effective, high % EE, and control release kinetic [20]. In the reported work, we have modified the Ch grafting with PF copolymer (ChPC), followed by to develop a self-assembly nanoparticles loaded with PD known as Pd-ChPC-NPs, to evaluate their safety and potential efficacy against DM.

Initially, we have modified PF with DMAP, TEA and succinic anhydride to formed monocarboxylate PF. This product was further used for grafting with CH in the presence of EDC and NHS. As shown in schematically Figure 1, ChPC was formed by the interaction of the primary amine group of Ch with the carboxyl group of monocarboxylate PF.
Figure 1: Chemical scheme for the synthesis of ChPC by coupling of PF onto Ch. The m and n stand for the variable units.

The final ChPC was evaluated by FTIR, as demonstrated in Figure 2. Briefly, Ch was showed OH group of stretching vibration bands (3357 cm\(^{-1}\)), C=O stretch vibration of amide I was confirmed by the band at 1651 cm\(^{-1}\), band at 1328 cm\(^{-1}\) correspond to C-N stretching of amide III. The band at 1560 cm\(^{-1}\) can be attributed to N-H bending (amide II band). The FTIR spectra of PF show the main absorption peak of aliphatic C-H stretching at around 2800-2900 cm\(^{-1}\). The O-H bends and C-O stretch was represented at band 1351 cm\(^{-1}\) and 1104 cm\(^{-1}\) respectively. The FTIR of ChPC shows that the sharpness of the peak was decreased and absence of the main peak at 1651 cm\(^{-1}\) in the final product respectively, compared to the pure polymers that were attributed to grafting of polymers.

Characterization of Pd-ChPC-NPs

The SEM and TEM morphological images of PD-ChPC-NPs were shown in Figures 3A and 3B respectively. These PD-ChPC-NPs images were represented the shape of NPs was uniform and smooth with average size around 250 nm without any aggregation, which directly related to stability of the NPs.

Meanwhile, the particles size and its distributions were evaluated by DLS, which also proves the poly-dispersed nature of resultant. The NPs size of ChPC-NPs and Pd-ChPC-NPs were analysed to be 199 ± 1.51 nm, and 263 ± 1.25 nm (Figure 4A) along with PDI of 0.146, and 0.162, respectively. However, the NPs size data recognized by DLS was quite similar to the data obtained by microscopic measurements. The higher % EE of PD-ChPC-NPs was found to be 86.49 ± 0.29, which help in suggesting their good DDS, and improved pharmacological activity in vivo.

The zeta potential was also measured using Malvern Zetasizer (Nano ZS, Malvern Instruments Corp, UK). The surface charge of ChPC-NPs and, Pd-ChPC-NPs were found to be 19.7 mV ± 3.13 mV and 12.9 mV ± 2.89 mV, respectively. PF is a non-charged amphiphilic polymer in nature, but when contacted with water, it will be converted into a negative charge, which may be caused by the ionization of the peripheral hydrophilic groups in the water molecules. Since Ch is a positively charged natural polymer, ChPC have obvious positive charges after being modified by Ch. The zeta potential was decreased in final product, which indicated that the
loading of PD was successful. At high temperatures (from 37°C and 43°C, respectively), the size of Pd-ChPC-NPs was decreased as the zeta potential was increased from ~15 mV to ~21 mV, respectively, which was attributed to electrostatic expulsion. In addition, at the higher temperature Ch could be protonated, thereby converting the primary group −NH₂ to −NH₃⁺ as a result dissociation of a water molecule from H₂O to H⁺ [21]. Due to Pd-ChPC-NPs have nanosized and higher zeta potential was supported the high stability, with sustained release kinetic [22].

**Stability study**

At all suitable temperatures (4 ± 2°C and 25 ± 5°C), the stability analysis of the Pd-ChPC-NP was evaluated within 6 weeks. The mean NPs size and PDI were evaluated at regular intervals. The analyzed results obtained by storage stability have been shown in **Figures 4B and 4C**. There was no significant difference between the initial and final results, which indicates that Pd-ChPC-NP was stable enough at different temperatures for 6 weeks according to ICH guidelines. This may be due to the fact that PF is a well-known pharmaceutical ingredient, which increases the solubility of the drug and improves its stability [23].

**In vitro release kinetic**

The in vitro release study of PD from Pd-ChPC-NPs by using USP dissolution test apparatus II at constant temperature and pH 6.8 for 12 h were summarized in **Figure 5A**. The release kinetic of PD from NPs was monitored using UV/Vis spectroscopy. As compared with PD, the release amount of PD from Pd-ChPC-NPs was 25% after 1 h and after 8 h, the cumulative release rate was significantly enhanced with 63% while 23% drug was released from free PD respectively. It is noted that the initial burst release type of PD maybe because of the dissociation of drug molecules from polymer matrix and the released result agrees to the previously reported studies of burst release of drug from PF-127-chitosan NPs dissolved in PBS at 8 h and followed the cumulative release over a period of 48 h [9,24]. The drug release from the polymer matrix at pH 6.8 is due to PF forms a rigid gel network at higher concentrations, which helps control PD release from NPs.

**% cell viability study**

The in vitro biocompatibility of PD-ChPC-NPs, ChPC-NPs and control (Free PD) were measured by using MTT assay. As displayed in **Figure 5B**, HT29 cell lines were incubated with NPs at a different PD equivalent concentration (0.001-10µg/ml). In particular, free PD exhibited a higher cytotoxic effect than PD-ChPC-NPs. In other words, it can be said that the killing potency of PD-ChPC-NPs was significantly higher than that of ChPC-NPs and free PD. The main reason may be that free PD could directly enter into the cell via passive diffusion and effective, whereas the cationic characteristic of NPs was mainly absorbed by negatively charged cell membrane via the endocytic pathway and then released and killed the cells. In addition, at the highest dose concentration, the % cell viability of the ChPC-NPs was ≥ 85% that indicated the protection and excellent biocompatibility potentials of the PD-ChPC-NPs for further in vivo pharmacological studies. This finding has consistent with previous reports in the literature [25].

**In vivo pharmacological activity**

Effect of NPs on BW: The initial and final BW of all treated groups was shown in **Figure 6A**. The results showed that the animals belonging to DG represented a significant weight loss on the last day (P < 0.001) compared with initial BW, which might be attributed that greater damage to proteins and fats [26].

On the other hand, D-PD-ChPC-NPs was shown significant weight gained (P < 0.001). According to the reported literature [5], we hypothesized that this may be due to the shielding of pancreatic islets, which support improved insulin secretion for good regulation of high blood sugar level. However, D-PD revealed a little weight gain on the last day (P ≥ 0.05). All final results were compared with D-MET oral administration.
Effect of NPs on biochemical markers

The in vivo anti-diabetes effects of all NPs were examined by measuring FBG levels in every week as displayed in Figure 6B. It was evaluated that DG significantly increased FBG levels (compared to NG, P < 0.001). In sharp contrast, FBI levels in DG was clearly reduced as represented in Figure 6C. The study found that compared with DG, the treatment of D-PD and D-PD-ChPC-NPs significantly reduced FBG levels, while the FBI of the same treatment group increased. D-ChPC-NPs did not show any hypoglycemic effect, indicating that PD-loaded NPs exert their hypoglycemic effect in diabetes animal models, which may be attributed to the insulin excretion and enhanced activity [5]. Thus, the results demonstrate that in vivo low blood glucose level seem to be completely consistent with the in vitro release kinetic analysis. The release profile of PD-ChPC-NPs shows the cumulative release pattern at pH 6.8, which may contribute to important blood sugar control in diabetic animals.

Moreover, Gly-Hb level was enhanced in the DG as compared with CG as displayed in Figure 6C. Compared with DG, PD-ChPC-NP significantly reduces Gly-Hb levels, which suggesting the anti-hyperglycemic characteristic of PD [27].

In patients with diabetes, high blood sugar levels are associated with dyslipidemia, which is directly related to heart attacks. Although, in the diabetic state, lipoprotein lipase is not activated due to insufficient insulin, but due to abnormal metabolism, hypertriglyceridemia and hypercholesterolemia have occurred [28].

Table 1: Effect of all NPs on Liver biomarkers (ALP, ALT, and AST) of DG.

<table>
<thead>
<tr>
<th>Animal group</th>
<th>AST (IU/L)</th>
<th>ALT (IU/L)</th>
<th>ALP (IU/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CG</td>
<td>90 ± 7.3</td>
<td>43.40 ± 2.51</td>
<td>84.34 ± 5.1</td>
</tr>
<tr>
<td>DG</td>
<td>155 ± 7.8</td>
<td>92.20 ± 5.6</td>
<td>211.23 ± 10.43</td>
</tr>
<tr>
<td>D-PD</td>
<td>149 ± 9.5§</td>
<td>70.32 ± 5.43§</td>
<td>151.2 ± 5.55§</td>
</tr>
<tr>
<td>D-PD-ChPC-NPs</td>
<td>103 ± 7.4§</td>
<td>54.31 ± 7.34§</td>
<td>126.12 ± 8.34§</td>
</tr>
<tr>
<td>D-ChPC-NPs</td>
<td>127 ± 8.4</td>
<td>90.10 ± 6.74</td>
<td>205.5 ± 7.21</td>
</tr>
</tbody>
</table>

Compared with CG, blood cholesterol and triglyceride levels in DG are significantly increased. While PD-ChPC-NPs (D-PD-ChPC-NPs) shows significantly decreased of blood cholesterol and triglyceride levels in diabetic animals models. Due to the low toxicity characteristics of PF, which contributed to be used as vehicles for sustained drug release pattern and effectively safe pharmaceuticals ingredient. Our current results are consistent with the published report [29].

Hyperglycemia can cause liver cell damage, resulting in more leakage of hepatic enzymes such as ALP, ALT, and AST into the serum [30]. Treatment with D-PD-ChPC-NPs shows a significant reduction of blood ALP, ALT, and AST levels as compared to DG as illustrated in Table 1. Our findings are consistent with studies in the literature based on in vivo pharmacological responses. As compared to PD and ChPC-NPs, PD-ChPC-NPs showed high anti-diabetic activities via prevention of pancreatic insulin secretion and enhancing insulin sensitivity (Figure 7).
Conclusion

In summary, modified by using FDA approved non-toxic polymer (PF) and natural biopolymer to form ChPC. FT-IR and zeta potential studies have confirmed successful use in ChPC. Due to its thermal effect on zeta potential and wall-permeability, ChPC has unique properties to encapsulate therapeutic agents (PD) for treating diseases. We have successfully prepared nano-sized PD-ChPC-NPs, characterized by SEM, TEM images with good stability at different temperatures. Compared with free PD, PD-ChPC-NPs can be assigned to cumulative-release characteristics, and excellent biocompatible with significant anti-diabetic efficacy. Taken together, our results indicate that PD-ChPC-NPs are potential drug candidates for the treatment of diabetes.

Conflict of Interest

The authors declare no conflict of financial interest.

References


