Amphiphilic dextran-vinyl laurate-based nanoparticles: formation, characterization, encapsulation, and cytotoxicity on human intestinal cell line

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Abstract

Dextran has been the model material for the therapeutic applications owing to its biodegradable and biocompatible properties, and the ability to be functionalized in variety of ways. In this study, the amphiphilic dextran was successfully synthesized through lipase-catalyzed transesterification between dextran and vinyl laurate. In aqueous solution, the produced dextran ester could self-assemble into spherical nanoparticles (“Dex-L NPs”) with approximately 200-nm diameter, and could incorporate porcine placenta hydrolysate with 60% encapsulation efficiency. Furthermore, Dex-L NPs exhibited low cytotoxic effects on human intestinal cell line and, thus, were potentially safe for oral administration. Taken together, the findings illustrate the potential of the newly developed nanoparticles to serve as an efficient and safe drug delivery system.

Keywords Dextran · Vinyl laurate · Nanoparticles · Drug delivery · Encapsulation · Cytotoxicity

Introduction

Dextran is a water-soluble homopolysaccharide of *D*-glucose, in which the monomers are linked through α -(1 → 6) linkages in the backbone and are branched with α -(1 → 4) linkages. Dextran’s biocompatibility and biodegradability has made it the polymer of choice in various applications, including pharmaceuticals, cosmetics, textile production, detergents, and biomedical therapeutics. In addition, certain derivatives of dextran possess the ability to self-assembly into nanoparticles that can assume diverse morphologies such as micellar, tubular, and spherical structures, with the sizes ranging from nano- to micro-meters. These dextran-based NPs can be employed as drug delivery carriers to shelter labile molecules from rapid degradation during transport

and to control their release (Wang et al. 2004). For instance, dextran-based polylactide (PLA) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE) nanoparticles (NPs) were developed for antitumor drug delivery (Wang et al. 2012). Molecules with diverse chemical and structural properties can be encapsulated in a similar manner, including bacteriocin (Kim et al. 2019); antisense oligonucleotides (Kiani et al. 2016); anticancer drug, doxorubicin (Li et al. 2013); antibiotics, amphotericin B (Tiyaboonchai and Limpeanchob 2007); and anti-rheumatic drug, methotrexate (Heo et al. 2017). In addition to protective capabilities, biocompatibility and biodegradability of dextran-based polymers are also highly desirable in the drug delivery systems, to ensure minimum cytotoxicity and unwanted accumulation in organs.

We hypothesized that attachment of hydrophobic groups to the dextran backbone should yield amphiphilic copolymer that can form nanoparticles through hydrophobic–hydrophilic interactions. Here, we report the synthesis and characterization of amphiphilic dextran-vinyl laurate-based polymer (Dex-L) and evaluate its efficacy as the encapsulation platform for porcine placenta hydrolysate. Porcine placenta extract has been widely reported to possess various therapeutic benefits, including enhancement of cell proliferation (Imamura et al. 2017), antioxidant activity (Tang et al.

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2013), and immunostimulation in pigs (Lee et al. 2013). However, oral application of porcine placenta hydrolysate is susceptible to protein degradation that can occur in the digestive system and will benefit from an effective encapsulation technology. For these reasons, we chose porcine placenta hydrolysate as the model cargo in the development of our new encapsulation strategy. To synthesize Dex-L, we employed lipase-catalyzed transesterification of dextran and vinyl laurate (VL). VL was selected as the acyl donor of hydrophobic groups due to its reported absence of toxicity and low solubility in polar solvent which should render the resulting particles more stable in aqueous solution (Kaewprapan et al. 2012, 2011; van Acker et al. 2015; Messinger and Bär 2014). The acquired polymer was then tested for its ability to self-assemble, and the characteristics of the resulting NPs including sizes and shapes were analyzed. Finally, as we would like to further apply the Dex-L-based NPs as a carrier for orally administered porcine placenta hydrolysate, the cytotoxic effects of NPs were preliminarily investigated in the colorectal adenocarcinoma (HT-29), as an in vitro model of human intestinal cells.

Materials and methods

Materials

Dextran T40 with average molecular weight of 40,000 g/mol, biosynthesized by *Leuconostoc mesenteroides*, was purchased from Pharmacia (Uppsala, Sweden). Lipase AY (EC 3.1.1.3) from *Candida rugosa* was obtained from Amano Enzyme Co. (Nagoya, Japan). VL, 18-crown-6, and dimethyl sulfoxide (DMSO, CHROMASOV®) was purchased from Sigma-Aldrich (St. Louis, US and Steinheim, Germany). The dialysis membrane with MWCO of 6000–8000 was purchased from Spectrum Laboratories Inc. (CA, USA). Protein hydrolysate of porcine placenta extract was prepared by trypsin digestion at 37 °C for 24 h, followed by heat inactivation and lyophilization as described in the previous study (Jung et al. 2014).

Preparation of modified dextran

Modified dextran (Dex-L) was prepared by lipase-mediated transesterification following the previously reported protocol (Kaewprapan et al. 2011). The pH-adjusted lipase with additional 18-crown-ether, prepared as described elsewhere (Kaewprapan et al. 2007), was used as the catalyst for transesterification in organic solvent. Briefly, the reaction was done by dissolving 81 mg of dextran T40 in 5 mL DMSO and stirring for 5 min at 50 °C. VL was then added to the dextran solution at different ratios and further stirred for

5 min before addition of lipase. The reaction was continued for 32 h at 50 °C, before each sample was allowed to cool at room temperature. Modified dextran samples were purified by dialysis against 85% ethanol, followed by 65% ethanol, and distilled water using a membrane with MWCO of 6000–8000. After that, the modified dextran samples were lyophilized for 48 h using freeze-dryer (Freeze One6 Plus, LABCONCO, USA).

¹HNMR analysis of modified dextran

¹HNMR spectra of Dex-L samples were recorded with a Bruker AVANCE 400 spectrometer (400 MHz) in DMSO-*d*₆. Based on the previous report (Kaewprapan et al. 2007), the peaks assigned were 4.91 (O–H4), 4.86 (O–H3), 4.66 (H1), 4.51 (O–H2), 3.73 (H6), 3.61 (H5), 3.49 (H6), 3.41 (H3), 3.2 (H2), 3.15 (H4), 2.29 (–CH₂–CO), 1.25 (–CH₂–CH₂–C O), 1.23 (–CH₂–, 8H), 0.84 (–CH₃). The degree of substitution (%DS) was calculated with the equation:

$$\%DS = \left(\frac{\frac{I_1}{3}}{\frac{I_1}{3} + \left(\frac{I_2 - I_1}{4} \right)} \right) \times 100, \quad (1)$$

where I_1 is the peak area at 0.84 ppm corresponding to the three protons of methyl group (–CH₃) on VL; I_2 is the integrated peak area between 4.51 and 4.91 ppm, corresponding to 9 protons of anomeric carbon (OH4, O–H3, O–H2, and H1) of dextran.

Dextran-based nanoparticle formation and encapsulation of protein hydrolysate

Dex-L nanoparticle was prepared as described in previous studies (Kaewprapan et al. 2012; Lavasanifar et al. 2002; Hornig and Heinze 2007) with slight modifications. Briefly, Dex-L sample was dissolved in 5 mL DMSO at the polymer concentrations of 1, 2, and 2.5 mg/mL. The sample was mixed by vortexing until homogeneity was achieved, and was then ultrasonicated for 3 min. Then, 5 mL of distilled water was added drop-wise into the Dex-L solution under vigorous magnetic stirring. Nanoparticle suspensions were transferred into a dialysis membrane with molecular weight cut-off of 6000–8000 and dialyzed against distilled water. The water was changed at least twice a day for 3 days for complete DMSO removal. A similar method was applied to the encapsulation of protein hydrolysate. Briefly, 12.5 mg of Dex-L was dissolved in 5 mL DMSO (or 2.5 mg/mL Dex-L). Porcine placenta protein hydrolysate was dissolved in filtered sterile water at various concentrations. Hydrolysate solution was added to the

Dex-L solution by drop-wise technique, followed by dialysis steps as described.

Stability of nanoparticle during storage

Dex-L nanoparticles with and without protein hydrolysate were prepared and stored at 4 °C for up to two months. The average size of nanoparticles in each sample was determined every week the during storage.

Size distribution measurement and morphological analysis of nanoparticles

Size distribution (Z-average size) and polydispersity index (PDI) of Dex-L nanoparticles were determined in filtered distilled water using a dynamic light scattering (DLS) particle size analyzer (Malvern Zeta Nanosizer ZS, Malvern Instruments Ltd). Size measurement was carried out at 25 °C with the scattering angle fixed at 90°.

The Dex-L nanoparticle samples were prepared on a carbon-coated copper grid and negatively stained with uranyl acetate solution (2.0 wt%) (Chiang et al. 2012). Transmission electron micrographs were acquired using a Hitachi HT7700 microscope operated at an accelerating voltage of 120 kV. For scanning electron microscopy, Dex-L nanoparticle was lyophilized and sputtered with platinum-palladium prior to visualizing by field emission scanning electron microscopy (Hitachi SU8010, Japan).

Determination of protein concentration using Lowry assay

Dex-L particle samples were freeze-dried and each powder was dissolved in DMSO at concentration of 1 mg powder/mL DMSO before subjected to Lowry assay (Lowry et al. 1951). BSA at various concentrations was used as a reference. BSA solution or suspension of samples (50 µL of each sample) was added to 96-well plate followed by 250 µL of Lowry's solution. The solution was mixed thoroughly by pipetting and incubated at room temperature for 10 min. Then, Folin–Ciocalteu's phenol reagent was mixed with the solution in each well. After 2 h, the absorbance of each sample was measured using microplate spectrophotometer (Thermo scientific multiskan GO) at wavelength of 750 nm.

Encapsulation efficiency (EE%)

The encapsulation efficiency of each sample was determined based on protein concentration using the formula (Wu et al. 2013):

$$EE\% = \frac{W_t}{W_i} \times 100, \quad (2)$$

where W_t is the total protein encapsulated in particle (µg); W_i is the initial protein in preparation step (µg).

Cytotoxicity test with HT-29 cell line

In this study, HT-29 human intestinal cell line obtained from ATCC® (Kettering Cancer Center with American Type Culture Collection) was used to study the cytotoxicity of Dex-L nanoparticles using MTT assay (Mosmann 1983). HT-29 cells were seeded onto 24-well plate at a cell density of 5×10^5 cells per well in DMEM medium with 4.5 g/L glucose, 4 mM L-Glutamine, and sodium pyruvate (Hyclone™, USA). The medium was supplemented with 10% Fetal Bovine Serum, 1% (v/v) 100× penicillin–streptomycin (Millipore) and 1% (v/v) of 100 mM Na-pyruvate (Millipore). The cells were incubated at 37 °C with 5% CO₂ prior to the experiment. The cells were then washed with PBS to remove the remaining culture medium and treated with new culture medium containing 0.0001, 0.001, 0.01, 0.1, and 1 mg/mL of Dex-L nanoparticles. HT-29 cells untreated with Dex-L was used as control experiment. After 24 h, each well was washed with PBS and incubated with 0.5 mg/mL of MTT substrate for 30 min. MTT solution was then removed and replaced with acidified isopropanol to dissolve the formazan product. The samples were transferred to a 96-well plate and the absorbance was analyzed using Multiskan™ GO Microplate Spectrophotometer (Thermofischer Life Science) at the wavelengths of 570 nm and 630 nm. The readout at 630 nm was subtracted from that at 570 nm to obtain the background-subtracted absorbance. The number of viable cells was estimated to be proportional to the quantity of formazan, as described by following equation:

$$\text{Cell viability (\%)} = \left(\frac{N_t}{N_c} \right) \times 100, \quad (3)$$

where N_t is absorbance of treated experiment and N_c is absorbance of control experiment.

Statistical analysis

Statistical data analysis was performed using SPSS version 18 (Statistical Package for the Social Sciences; SPSS Inc. Released 2009. PASW Statistics for Windows, Version 18.0. Chicago: SPSS Inc). The significant difference between control and treated cells was analyzed by one-way ANOVA with post hoc Tukey HSD at the confidence level of 95% (p value < 0.05).

Results and discussion

Transesterification of dextran and vinyl laurate to obtain amphiphilic dextran

Dextran T40 was modified by lipase-catalyzed transesterification (Fig. 1) with different ratios of dextran and vinyl laurate. The modification of dextran was confirmed by ^1H NMR spectroscopy (Fig. 2), and the degree of substitution (% DS) in each condition is shown in Table 1. As expected, the pH-imprinted lipase with 18-crown-ether is the most effective catalyst under this experimental condition and the results were in agreement with the previous study (Kaewprapan et al. 2007). According to the data, the two highest degrees of substitution (approximately 72% and 67%) were achieved in conditions with the dextran T40:VL ratio of 1:4 and the pH-adjusted lipase co-lyophilized with 18-crown-6 ether as catalyst (sample number 6 and 8). Interestingly, the amount of VL in a reaction affected the transesterification more than that of lipase. For instance, when compared with sample 5, the degree of substitution in sample 6 was higher by 18%, whereas only 13% increase in sample 7 was observed, suggesting that VL could be the main limiting factor. Further study should be performed to investigate the stability of lipase and determine the maximum amount of VL that can be added to a given dextran.

Nanoparticle formation

In a previous study, dextran-vinyl decanoate copolymer (Dex-D) was synthesized and shown to exhibit slight solubility in water. Compared to Dex-D, however, the solubility of Dex-L in water was significantly lower (data not shown). Therefore, DMSO was used to solubilize Dex-L, and subsequent removal of the organic phase was achieved by dialysis instead of the evaporation technique that was used in the preparation of Dex-D NPs. The Z-average size and polydispersity index of Dex-L particles were 211.15 ± 3.92 nm and 0.205 ± 0.048 , respectively (Fig. 3). Transmission electron microscopy revealed spherical particles with approximately 200-nm diameter, indicating that Dex-L NPs were successfully prepared using our workflow (Fig. 4). Additional validation with scanning electron microscopy (Fig. 5) also showed particles with sizes and morphology, similar to those observed under DLS and TEM. It should be noted that the surface of NPs appeared rugged under SEM. However, the observed roughness might not represent the actual surface of NPs, but rather the artifact from metal coating that was performed as part of SEM sample preparation.

Effect of polymer concentration on the Dex-L nanoparticle size

To elucidate the effect of polymer concentration on the size of nanoparticle, the Z-averages of Dex-L at various polymer concentrations (1, 2, and 2.5 mg/mL) were determined by DLS (Table 2). Strikingly, the highest

Fig. 1 Proposed transesterification of dextran and vinyl laurate. The reaction was performed in DMSO at 50 °C with lipase as a catalyst

Fig. 2 ^1H NMR spectra (400 MHz) in DMSO-d_6 of dextran T40 (a), vinyl laurate (b), and Dex-L (sample 8) (c). Peak I1 corresponds to the three protons of methyl group ($-\text{CH}_3$) on VL, whereas I2 corresponds to 9 protons of anomeric carbon (OH4, O-H3, O-H2, and H1) of dextran

Table 1 Conditions used in transesterification for modification of dextran

#	Reaction condition	Lipase (mg)	Ratio of DexT40: VL ^a	% DS
1	No lipase	–	1: 2	1.05
2	No lipase	–	1: 4	4.77
3	pH-adjusted lipase	26	1: 2	25.76
4	pH-adjusted lipase	26	1: 4	30.53
5	pH-adjusted lipase co-lyophilized with 18-crown-6 ether	26	1: 2	49.97
6	pH-adjusted lipase co-lyophilized with 18-crown-6 ether	26	1: 4	67.73
7	pH-adjusted lipase co-lyophilized with 18-crown-6 ether	52	1: 2	63.70
8	pH-adjusted lipase co-lyophilized with 18-crown-6 ether	52	1: 4	71.55

^aDexT40:VL is the ratio between the weight of dextran T40 and vinyl laurate

Fig. 3 Size distribution of Dex-L nanoparticle measured by dynamic light scattering

concentration of polymer (2.5 mg/mL) was found to yield the smallest particle size, in contrast to the general notion that particle size should increase with building block concentration. The inverse correlation observed may be explained by the presence of Dex-L with low %DS, which had higher surfactant activity than the more highly substituted counterpart. As Dex-L in this work was prepared without size-based fractionation, the product was a mixture of polymers at various %DS, consisting of large amount of highly-substituted Dex-L and minor amount of low-substituted polymer. Thus, the use of Dex-L at high concentration would unavoidably introduce a larger amount of low-substituted Dex-L into the solution, and in turn increase surfactant activity that hinders coalescence.

Fig. 4 Morphology of Dex-L nanoparticle under transmission electron microscopy at 120,000 V with magnification of 2500× (left) and at 9600× (right)

Fig. 5 Morphology of Dex-L nanoparticle under scanning electron microscopy with accelerating voltage of 5.0 kV (left) and 15.0 kV (right)

Table 2 Effect of polymer concentration on the particle size and polydispersity index (PDI) of Dex-L nanoparticles ($n = 3$)

Sample	Dex-L concentration (mg/mL)	Size distribution	
		Z-average (nm)	PDI
1	1.0	195.00 ± 2.26	0.403 ± 0.006
2	2.0	180.23 ± 1.45	0.037 ± 0.020
3	2.5	160.93 ± 2.11	0.007 ± 0.003

The observed trend agrees with a previous report that increase in surfactant concentration leads to smaller PLGA-based nanoparticles (Ray et al. 2015).

Encapsulation of protein hydrolysate

According to the result shown in Fig. 6, protein hydrolysate concentration affected the size distribution of Dex-L NPs. When the hydrolysate concentration was raised, the size distribution and PDI of Dex-L particle also increased. A similar trend has been observed in a previous study (Zhang et al. 2019), in which encapsulation of α -tocopherol resulted in the enlarged particles. The initial cargo concentration of 5 mg/mL yielded the highest encapsulation efficiency (Fig. 7), albeit lower than the efficiency reported in a previous work where BSA was encapsulated in dextran-PEG (Wu et al. 2013). This discrepancy may be attributed to the fact that the average protein size of the hydrolysate is smaller than BSA (data not shown).

Fig. 6 Average size and polydispersity index of Dex-L at various concentrations of protein hydrolysate

Fig. 7 Encapsulation efficiency of Dex-L at various concentrations of initial protein hydrolysate

Evaluation of cytotoxic effects of Dex-L nanoparticles on cultured HT-29

Despite reported low cytotoxicity of dextran and vinyl laurate when evaluated separately, we would also like to ensure that their copolymer, and the nanoparticles thereof, did not exhibit unanticipated toxic effects. As the encapsulated placenta hydrolysate was intended for oral administration, we chose the colorectal adenocarcinoma cells HT-29, commonly used as a model for digestive tract cells, as the testbed. The cell viability assessment by the MTT assay using the various concentrations of Dex-L nanoparticles revealed that, compared to the untreated control, cell viability of HT-29 at 0.0001, 0.001, 0.01, 0.1, and 1 mg/mL was $103.15 \pm 10.01\%$, $85.75 \pm 12.02\%$, $93.36 \pm 8.48\%$, $92.06 \pm 10.68\%$, and $87.13 \pm 9.08\%$, respectively (Fig. 8). No significant difference was observed among treatment groups and with the control experiment ($p < 0.05$), suggesting that Dex-L nanoparticles had negligible effect on the cell viability of HT-29. This result was in accordance with the previous report where dextran by itself did not perturb cell viability of HT-29 (Kothari et al. 2015).

Stability of Dex-L NPs during storage

Dex-L NPs with and without porcine placenta hydrolysate were stored at 4 °C for eight weeks to evaluate their stability during storage. The samples were analyzed every week with DLS to monitor any changes in particle size that might have occurred over time. As shown in Fig. 9, the size of Dex-L NPs remained relatively constant over the course of storage,

Fig. 8 Effect of Dex-L nanoparticle at various concentrations on cell viability of HT-29. Values are the mean $\pm$ SD ($n=5$). The statistically significant differences between treatments and untreated control were determined using one-way ANOVA (at p value < 0.05)

Fig. 9 The average size of Dex-L nanoparticles during storage at 4 °C up to 2 months. The size of Dex-L nanoparticles with (dashed line) and without porcine placenta hydrolysate (solid line) were determined every week using dynamic light scattering

suggesting that the complexes were highly stable. Furthermore, the absence of significant changes in Dex-L NPs containing the protein hydrolysate indicates that the encapsulation of this cargo did not destabilize the assembly.

Conclusions

Dextran can be modified to enable self-assembly into NPs, by grafting of hydrophobic moieties onto the dextran backbone. In this work, we have developed the method for preparing Dex-L nanoparticles using the amphiphilic dextran obtained from lipase-catalyzed transesterification of dextran and vinyl laurate. Without cargo, the Dex-L NPs generated were spherical and roughly 200-nm in diameter. The NPs were able to encapsulate porcine placenta hydrolysate with approximately 60% packaging efficiency and the particle diameter was enlarged to 300 nm as a result. In addition, Dex-L NPs at concentrations between 0.0001 and 1 mg/mL were not cytotoxic to HT-29 cells. Collectively, our findings demonstrate that Dex-L is a tractable, robust and non-toxic alternative as a carrier for orally administered molecules. Further studies on Dex-L NPs should investigate their drug release characteristics, efficiency of cargo delivery, as well as ability to encapsulate other hydrophilic and hydrophobic compounds.

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