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Reverse micelles based on β-cyclodextrin-incorporated amphiphilic polyurethane copolymers for protein delivery

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A series of amphiphilic polyurethane (PU) copolymers were synthesized by condensation reaction of poly(ethylene glycol) (PEG) of different molecular weights and 1,6-hexamethylene diisocyanate (HDI), with/without end-capped heptakis(2,6-di-O-methyl)-β-cyclodextrin (DM-β-CD). Their chemical structures were characterized by Fourier transform infrared (FT-IR) spectroscopy and proton nuclear magnetic resonance (^{1}H NMR) spectroscopy. Their molecular weights, thermal properties and crystallization properties were investigated using gel permeation chromatography (GPC) and differential scanning calorimetry (DSC), respectively. A model protein, bovine serum albumin (BSA), was encapsulated into the PU reverse micelles (RMs) with/without DM-β-CD entities using emulsification method in dichloromethane (DCM), and then further transferred in biocompatible oil, *i.e.*, ethyl oleate. The diameter of RMs in DCM decreased from 180 – 480 nm to 100 – 280 nm upon heating, as determined by dynamic light scattering (DLS), and it was spherical in shape, as observed by scanning electron microscope (SEM). The encapsulation efficiency (EE) and loading capacity (LC) of BSA in the RMs composed of DM-β-CD-contained PUs were much higher than those without DM-β-CD. *In vitro* release studies showed that the release rate of RMs of DM-β-CD-contained PUs was faster than their counter parts without DM-β-CD. Interestingly, among all the RMs in the present study, the RMs of DM-β-CD-contained PU composed of the irregular segments of both PEG1000 and PEG2000 exhibited the highest EE and LC, and the fastest release rate of its cargo. These results highlight the ability of RMs of proper PUs composition to act as carriers for protein in an oleous phase with good EE and proper release behavior, paving a new way for the application of PU-based RMs in protein or peptide delivery.

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Introduction

Polyurethanes (PUs) as a special class of biomaterials have been widely used in drug delivery systems (DDS) for controlled drug release, tissue engineering scaffolds, artificial muscles, etc., due to their attractive physical properties and good biocompatibility.[1-6] Polyurethanes could be easily synthesized with diisocyanate and polyalcohol precursors through a condensation reaction.[7] The performance properties of PUs can be precisely and extensively modified by selecting appropriate raw materials, catalysts and auxiliary compounds to satisfy their applications.

Numerous formulations have been developed when using polymers as drug carriers.[8,9] Among them, polymeric micelles are widely used, which defined as core-shell structures through the self-assembly of amphiphilic polymers to form in a solvent which is considered hostile towards either moiety. In water, these micelles are characterized as a hydrophobic core shielded from the external medium by a hydrophilic shell. These normal micelles have been extensively studied in terms of their ability to improve the aqueous solubility of hydrophobic therapeutic agents.[10,11] In contrast, much less studies have been done on the micelle formation in organic solvent. Theoretically, the self-association of amphiphilic copolymers can yield nanostructures with a polar core and a hydrophobic shell in non-aqueous solvents. Such assemblies are commonly referred to as reverse micelles (RMs) to differentiate them from the micellar aggregates formed in aqueous media. RMs was usually constructed unimolecularly based on dendrimers, branching

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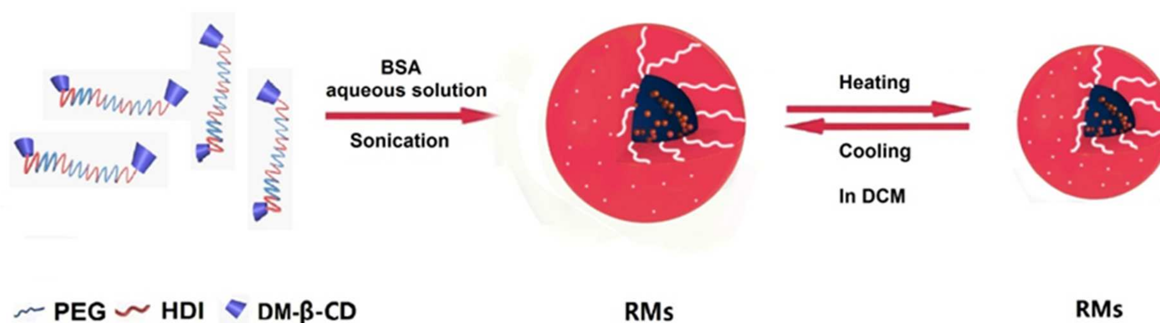

Fig. 1 Schematic illustration of RMs formation and the process of the core shrink upon heating and cooling.

polymers.[12,13] In the late 1990's, two research groups focused on the assembly of block ionomers in toluene, carbon tetrachloride and cyclohexane.[14-16] Recently, Krauel and et al. prepared poly(alkylcyanoacrylate) RMs using water-in-oil microemulsion technique.[17] Thayumanavan et al. reported that poly(styrene-*co*-acrylic acid) block copolymers could form invertible amphiphilic homopolymers, and polymers with such properties could find use in applications such as carriers for trafficking drugs through the lipid bilayers.[18] RMs from amphiphilic homopolymers with carboxylic acid and quaternary amine substituents were used to selectively enrich biomarker peptides and protein fragments from human serum.[19] Vachetet et al. had shown that RMs from amphiphilic homopolymers and their dendritic analogues can selectively extract/fractionate peptides based on their isoelectric points for direct MALDI-MS detection.[20] However, the application of RMs in drug delivery is rather rare. The RMs could transferred protein into the oil phase, while oil phases can form a continuum with the lipid barriers in the body, such as cell membranes and skin lipids, and in this way might allow passage of dissolved components which would otherwise be excluded.[21] We and Leroux et al. reported the construction of RMs based on linear and star-shaped alkylated poly(glycerol methacrylate)s (PGOHMAs) for peptide and protein delivery.[22,23] But their drug loading ability is limited and in urgent needs to be improved.

On the other hand, cyclodextrins (CDs) are a series of polyhydroxy compounds widely used for drug delivery. They possess different cavity size, low toxicity, certain hydrophilicity, and sometimes protection effect of the included/conjugated drugs from deactivation.[24] β-CD consists of seven D-glucopyranose residues linked by α-1,4-glycosidic bond, and has been introduced into PUs. For instance, Yang et al. synthesized a series of thermogelling copolymers composed of heptakis(2,6-di-O-methyl)-β-CD (DM-β-CD), poly(propylene glycol) (PPG), and poly(ethylene glycol) (PEG).[25] Cesteros et al. synthesized hydrophilic PUs networks based on poly(ethylene glycol) and β-CD.[26] There are 21 hydroxyl groups on β-CD but 7 on DM-β-CD. Too much hydroxyl groups may offer more crosslinking points. The polymers bearing too much crosslinking points will tend to form gel instead of nanoparticles. Therefore, DM-β-CD was used in this study. To the best of our knowledge, PUs have neither been used to form RMs in organic solvents nor used as vehicles for protein delivery. We hypothesized that PUs with proper structure and molecular weight could form tuneable RMs in the oil phase for protein delivery. Moreover, introducing DM-β-CD in PUs might further enhance their protein loading capacity (LC) because of the interaction between CD and protein.[27]

Our previous study has shown that 1,6-hexamethylene diisocyanate (HDI)-based PUs possessed good cell viability.[28] In this study, a series of high molecular weight and low molecular weight PUs based on HDI and PEG of different molecular weights were synthesized by a condensation reaction between diol and diisocyanate, and end-capped with DM-β-CDs. The RMs could be obtained based on the PUs to solubilize a model protein, *i.e.*, bovine serum albumin (BSA), in dichloromethane (DCM) and ethyl oleate (Fig. 1). Their drug loading and release behavior were compared with PUs without DM-β-CD. It is expected that the CD-contained RMs could accommodate the protein in oil with improved encapsulation efficiency (EE) and LC, and exhibited different drug release behavior.

Experimental

Materials

PEG (Mw = 1000 or 2000) and DM-β-CD were purchased from Aladdin Co. (Shanghai, China). Purification of the PEG was performed by dissolution in DCM followed by precipitation in diethyl ether and drying in vacuum before use. Hexamethylene diisocyanate (HDI, 98%) and dibutyltindilaurate (95%) were purchased from Alfa Co. (Tianjin, China). BSA was bought from Aladdin reagent Co. Ltd (Shanghai, China). All other reagents were obtained from Tianjin Chemical Reagent Co. (Tianjin, China) and used without further purification.^{1}H NMR spectra were recorded on a Bruker AV-400 spectrometer (400 MHz, Bruker, Freemont, CA). Samples were dissolved in deuterated chloroform, or deuterated water. Gel permeation chromatography (GPC) measurements were performed in THF,

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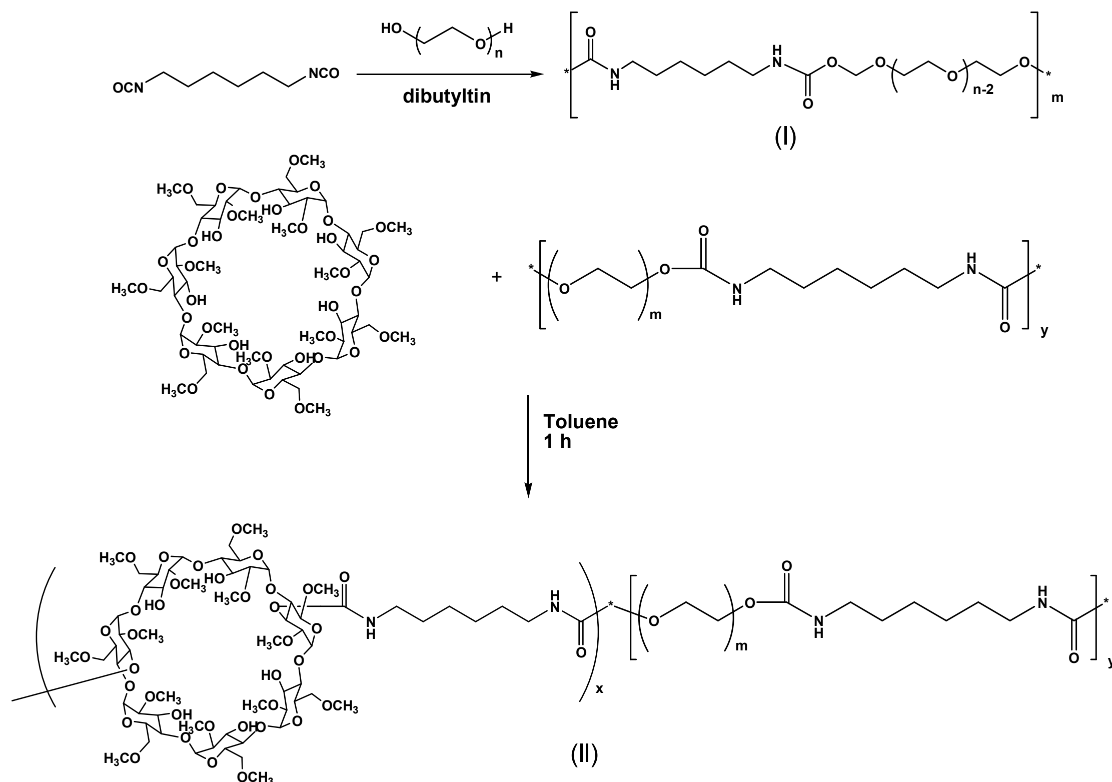

Sheme 1. Synthesis of PEG-HDI (I) and PEG-HDI-β-CD (II).

using a Waters 2414 system (Milford, MA) equipped with a refractive index detector. Adequate molecular weight separation was achieved using three Waters Styragel columns (HT3, HT4, HT5) in series at a flow rate of 1.0 mL min^{-1} and a temperature of 35 °C. Calibration curves were obtained with nearly monodisperse polystyrene.

Synthesis of polyurethane

PEG-based polyurethanes (PUs) were synthesized according to previous reports[26-29] with modification (Scheme 1). Briefly, PEG1000 (1.995 g, 1.995 mmol) and DM-β-CD (0.250 g, 0.19 mmol) were dried at 70 °C in vacuum overnight. PEG1000 (1.995 g, 1.995 mmol) was dissolved in 1, 2-dichloroethane (DCE) and heated at 110 °C. Residual water was removed by azeotropic distillation. After cooling to 70 °C, HDI (0.369 g, 2.1945 mmol) was added to this solution. Dibutyltindilaurate (0.5 wt%, 14 μL or 28 μL, with respect to the reactant) in dried DCE was added to the solution as a catalyst. The mixture was stirred at 70 °C for 8 h or 16 h under dry nitrogen. Finally, an excess amount of DM-β-CD (0.25 g, 0.19 mmol) dissolved in toluene was added into the reaction mixture and stirred for another hour at 70 °C. The resulting PU was poured into 200 mL of Et_2O. The precipitate was washed 3 times with Et_2O, and dried for 24 h at 40 °C under vacuum. The production was then dissolved in 30 mL DMF, dialyzed (MW cut-off of 7 KDa) against distilled water for three days in order to remove excess DM-β-CD, followed by lyophilization. As for PEG1000&2000-HDI, PU containing both PEG 1000 and PEG 2000, PEG1000 (0.998g, 0.998mmol) and PEG2000 (1.995 g, 0.995 mmol) were mixed and dried at 70 °C in vacuum overnight. Using the above method, a series of PEG-HDI were synthesized as a contrast without addition of DM-β-CD.

Thermal analysis

The thermogravimetry analysis (TGA) of the PUs was carried out under nitrogen atmosphere with a heating rate at 10 °C/min using a thermogravimetric analyzer (Netzsch TG209). Differential scanning calorimetry (DSC) analysis was performed with a differential scanning calorimeter (Netzsch PC-200). Specimens of 3-5 mg were encapsulated in aluminium pans and heated at a heating rate of 10 °C min^{-1}; cooled to -60 °C at a cooling rate of 10 °C min^{-1} and kept at -60 °C for 3 min; the samples were heated again at a heating rate of 10 °C min^{-1} to 160 °C. The DSC thermograms of samples were recorded during the second heating run process.

Preparation of RMs

The polymer (4 mg) was dissolved in dichloromethane (DCM, 4 mL), followed by addition of 40 μL aqueous solution of BSA (100 mg/mL).[19,20] The mixture was sonicated (SONICS, 3s on, 2s off) until homogeneous emulsification formed. A clear solution was obtained after stirring for 4 to 5 hours, and polymeric RMs in DCM were formed. For preparation of oleaginous micellar solutions, ethyl oleate (2 mL) was added into the above DCM solution, and the organic solvents

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evaporated by evacuation. All solutions were then diluted to a final polymer concentration of 1 mg/mL.[22]

Dynamic light scattering (DLS)

The mean hydrodynamic diameter and polydispersity index (PDI) of the RMs were determined at various temperatures on a Zetasizer Nano ZS90 (Malvern Instruments, Southborough, MA). The temperature interval was configured at 5 °C ranging from 20 °C to 35 °C in DCM, from 25 °C to 45 °C in ethyl oleate, and equilibrated for10 min before each measurement.

Variable temperature ^{1}H NMR

Variable temperature ^{1}H NMR (400 MHz) spectra were recorded on a thermo-regulated Bruker Avance 400. At each temperature, the solutions or suspensions were equilibrated for 20 min before measurement.

Scanning electronmicroscope (SEM)

RMs solution was dropped on a cover slip. After evaporation of organic solvent, the samples were coated with a thin gold layer. The morphology of RMs was observed on JSM-6700F type field emission SEM (JEOL, South Korea) and scanned at an accelerated voltage of 10 kV.

Transmission electronmicroscope (TEM)

TEM was conducted on a Jeol instrument (JEOL1400, Japan) with an accelerating voltage of 100 kV. Samples were prepared by dropping the RM (0.1 mg/mL) onto a carbon coated copper grid.

Determination of DM-β-CD content in PUs

A fixed volume (0.1 mL) of liquified phenol (80% w/w) was added to the PEG-HDI-CD polymer aqueous solution (1 mg/mL, 2.5 mL) followed by addition of concentrated sulphuric acid (98%, 5 mL). Its UV absorbance was measured at 490 nm (UV-3310) against an appropriate blank. The concentration of DM-β-CD was determined from a standard curve of DM-β-CD solution.[30] The carbohydrates will appear to coloration with the phenol-sulphuric acid.[31] The number of cyclodextrin in the polymer unit was calculated using the following equation:

$$\text{Molar ratio of DM-}\beta\text{-CD (100\%)} = \frac{\text{The number of moles of DM-}\beta\text{-CD in the polymer}}{\text{The number of moles of polymer}} \times 100\%$$

Determination of RMs loading

The amount of BSA encapsulated in RMs was determined by recovering the protein from the RMs. Acetone was added to the ethyl oleate solution to dissolve the polymer. The polymers in the supernatant were removed by centrifugation three times. The remaining protein pellet was air dried and dissolved in distilled water.[32] The BSA content was determined by the Coomassie Blue method using a UV spectrophotometer at a wavelength of 590 nm.[33] The LC and EE were calculated according to the following equations:

$$\text{EE (\%)} = \frac{\text{Final loading}}{\text{Initial loading}} \times 100\%$$

$$\text{LC (\%w/w)} = \frac{\text{Mass of loaded guest}}{\text{Mass of nanoparticles}} \times 100\%$$

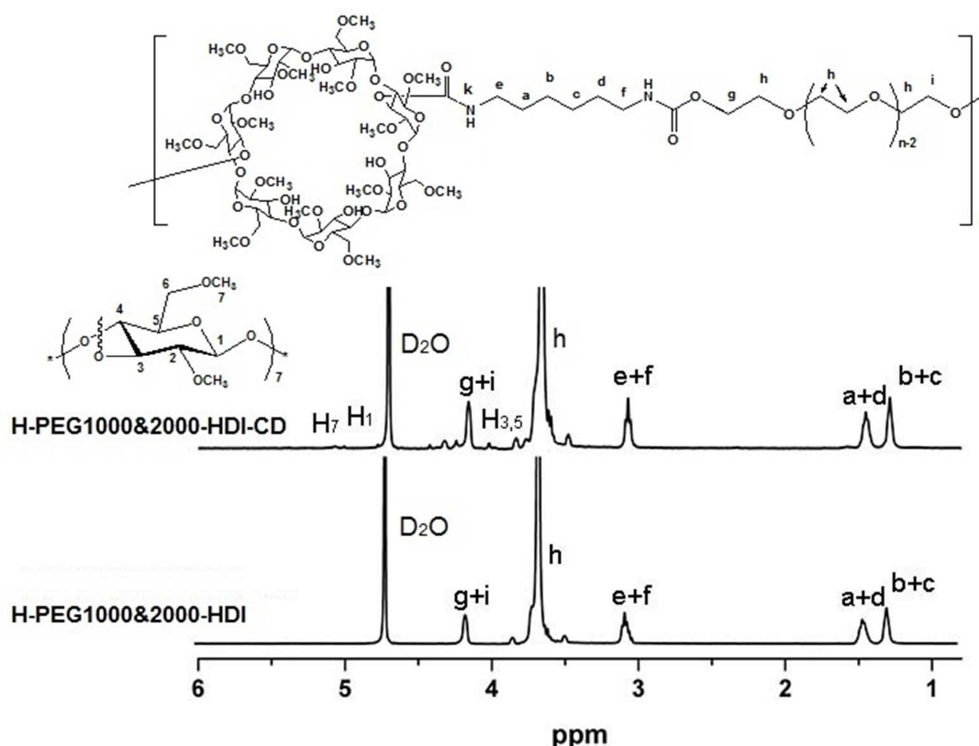

Fig. 2 ^{1}HNMR spectra of H-PEG1000&2000-HDI-CD and H-PEG1000&2000-HDI in D_2O.

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Table 1. Characteristics of PUs.

PUs	Mn	PDI	DCM	Ethyl oleate	Content of DM-β-CD[a] (%)
			Diameter (nm)	Diameter (nm)	
H-PEG1000-HDI-CD	69100	2.12	397.0	390.0	65.6
H-PEG1000-HDI	59000	2.17	493.0	482.0	-
H-PEG1000&2000-HDI-CD	66200	2.17	337.8	358.1	4.2
H-PEG1000&2000-HDI	70400	1.96	246.2	250.0	-
H-PEG2000-HDI-CD	54600	1.71	255.0	272.2	2.0
H-PEG2000-HDI	66400	1.82	310.6	325.8	-
L-PEG1000-HDI-CD	10800	1.42	443.0	_	20.1
L-PEG1000-HDI	19200	1.69	480.0	_	-
L-PEG1000&2000-HDI-CD	22600	1.30	303.1	_	1.3
L-PEG1000&2000-HDI	31400	1.38	198.0	_	-
L-PEG2000-HDI-CD	27200	1.35	243.9	_	1.1
L-PEG2000-HDI	25100	1.66	272.3	_	-

[a] Calculated using phenol-sulphuric acid method and expressed as the percent molar ratio of DM-β-CD unit to polymer unit.

The transfer rate of BSA from RMs in DCM to water

The transfer rate of BSA from DCM to aqueous solution was evaluated at room temperature (25 °C). The distilled water (2 mL) was added to the equal volume of RMs solution (1 mg/mL) in DCM in a capped beaker. At every designated interval, distilled water (1 mL) was taken out and fresh distilled water (1 mL) was replenished to keep a constant volume. The amount of BSA released into the distilled water was determined by the Coomassie Blue method. The concentration of BSA released from the RMs was expressed as a percentage of the total BSA available and plotted as a function of time. The cumulative BSA release was calculated through the equation below:

$$\text{Cumulative BSA transfer rate (\%)} = \frac{Mt}{M\infty} \times 100$$

Where Mt is the amount of drug released from RMs at time t and $M\infty$ is the amount of drug released from the RMs at time infinity.

In vitro release kinetics of BSA from RMs in ethyl oleate

Distilled water (2 mL) was added to an equal volume of oleaginous micellar solutions (1 mg/mL) of ethyl oleate in a capped beaker at 37 °C. The methods of sampling and quantification were the same as above.

Circular dichroism spectra of BSA samples

The circular dichroism measurements of free BSA in the release medium and control BSA solutions in water were performed on a Jasco-715 Spectro polarimeter at 20 °C using the matched 10-mm path length quartz cells. Each sample solution was scanned in the range of 190-250 nm. A circular dichroism spectrum was recorded as the average value of three scans.

Results and discussion

Synthesis and characterization of PUs

Both PEG-HDI-CD and PEG-HDI series PUs were synthesized by a condensation reaction of HDI and PEG (molecular weight of 1000 and 2000) with or without addition of the end-capped DM-β-CD (Scheme 1). Gels were formed when DM-β-CD was added at the beginning of the reaction, indicating that the cross-linking occurred. The gel couldn't be used for preparation of RMs. We thus optimized the reaction. After condensation reaction of HDI and PEG for 8 h, excess amount of DM-β-CD

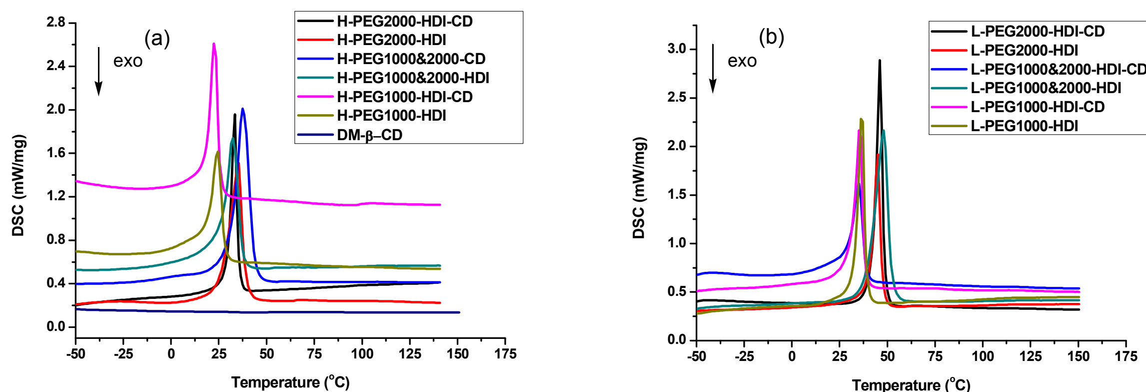

Fig. 3 DSC thermograms of H-Mw PUs, DM-β-CD (a) and L-Mw PUs (b).

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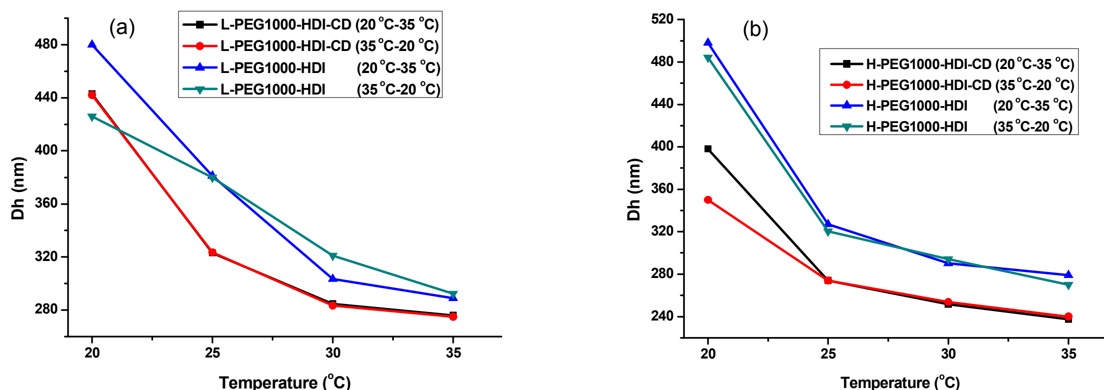

Fig. 4 Temperature dependence of the diameter of RMs in DCM solution.

was added and stirred for another hour at 70 °C to avoid the cross-linking reaction. All the PUs used in this study was not in gel state, indicating no or only a slight cross-linking occurred. The PUs were consisted of HDI as hydrophobic segments, and PEG with/without DM-β-CD as hydrophilic segments. The chemical structures of the resulting polymers were characterized by ^{1}H NMR and FT-IR. Fig. 2 shows the ^{1}HNMR spectra of PEG1000&2000-HDI with/without CD. The peaks of a+d, b+c and e+f at 1.5 ppm, 1.34 ppm and 3.0 ppm were assigned to the methylene protons of HDI. The peaks of g+i and h at 4.19 ppm and 3.6 ppm were assigned to the methylene protons of PEG. The peaks O(3)H, H(1) at 5.0 ppm, 4.97 ppm , and H(3), H(6), H(5), H(2) and H(4) at 3.28-3.87 ppm were assigned to the methylene protons of DM-β-CD. The molecular weight, polydispersity index (PDI) and content of DM-β-CD of polyurethanes are given in Table 1. Low-molecular weight (L-Mw) PUs were obtained after reaction for 8 h upon addition of 14 μL of catalyst, and are defined as L-PEG-HDI, while high-molecular weight (H-Mw) PUs were obtained by extension of reaction time to 16 h with addition of 28 μL of catalyst, and are defined as H-PEG-HDI.

DSC was carried out to obtain the thermo-properties of PEG-HDI-CD and PEG-HDI series, as well as DM-β-CD (Figure 3). As shown in Fig. 3a, DM-β-CD does not show any thermal transitions during the course of heating. The introduction of DM-β-CD into the PUs has no significant effect on Tm. All the PUs showed a clear melting temperature (Tm). The Tm of H-Mw PUs was around 24 – 38 °C, and that of L-Mw PUs was around 33 – 50 °C. The Tm values of H-Mw PUs (Fig. 3a) are lower than that of L-Mw PUs (Fig. 3b). The chain mobility is one of an important factor for crystallization. The increase in MW probably facilitated the construction of regular structure, and the formation of crystal phase. Additionally, the Tm increased with the increase of PEG molecular weight in H-Mw polymers, for instance, it was 25 °C for PEG1000-HDI-CD/PEG1000-HDI, and 37 °C for PEG2000-HDI-CD/PEG2000-HDI. The increased chain length of PEG was contributed to the increased size of the crystallites, resulting in the increase of the Tm.[34] Longer soft segment in H-Mw polymers enhances the extent of inter- or inner-molecular hydrogen bonding of this type of segmented PUs.[35] The Tm of PEG1000&2000-HDI-CD and PEG1000&2000-HDI were 38 °C and 35 °C. The irregular PEG segment was not favorable for the construction of regular structure. The Tm of L-Mw polymers showed a similar trend to that of H-Mw PUs (Fig. 3b).

Temperature sensitivity of PUs

The size of the RMs was determined by DLS in DCM and ethyl oleate (Table 1). The chain length of PEG affected the size of RMs. The longer chain length of PEG led to the smaller size. Even though DM-β-CD-contained PEG1000-HDI and PEG2000-HDI exhibited high drug loading, the size of RMs was smaller than polymers without DM-β-CD. Introduction of CD will endow the polymer-enhanced hydrophilicity, which will enhance the driving force for the formation of compact RMs. It was reported that, in aqueous solutions[36], the hydrophobic segment could promote the core compactness of normal micelles, and enlarged hydrophobic portion resulted in the formation of smaller particles. Similarly, the larger hydrophilic portion can promote the compactness of the micellecores in organic solutions. Therefore, an increased hydrophilic segment resulted in reduced particle size. However,

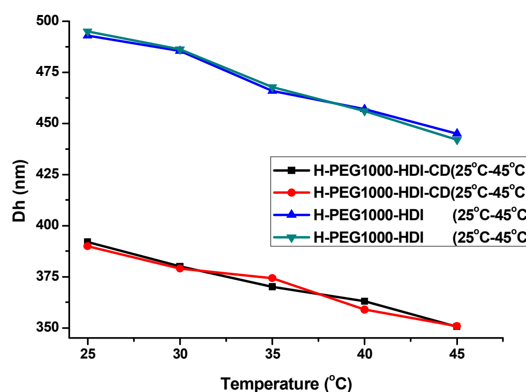

Fig. 5 Temperature dependence of the RMs diameter in ethyl oleate.

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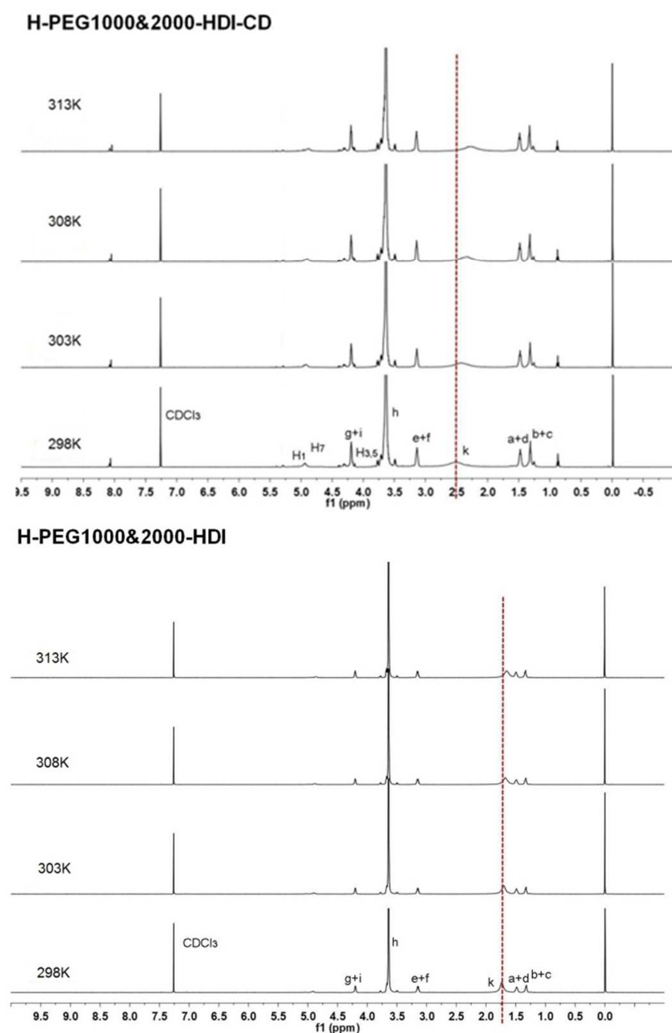

Fig. 6 Variable temperature ^{1}H NMR of H-PEG1000&2000-HDI-CD and H-PEG1000&2000-HDI.

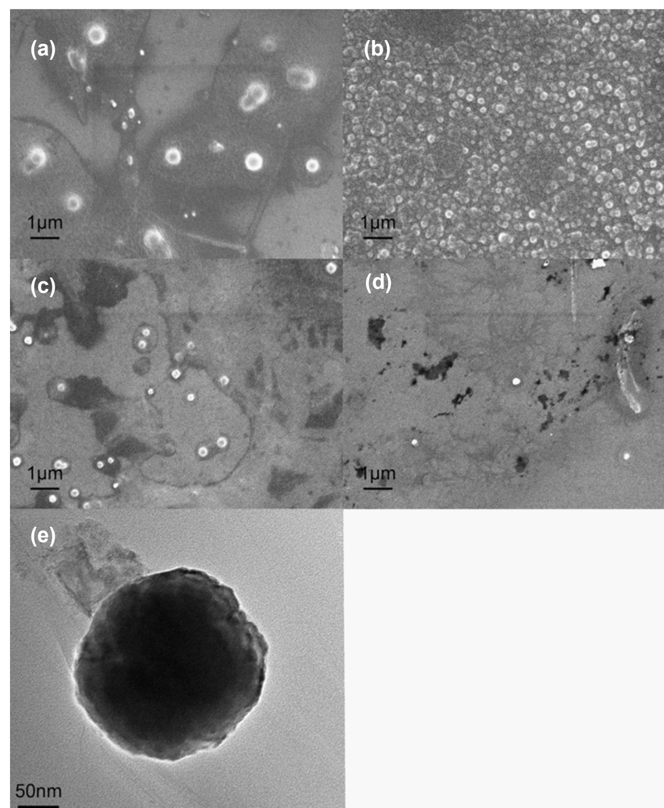

Fig. 7 SEM images of H-PEG1000&2000-HDI-CD (a), H-PEG1000&2000-HDI (b), L-PEG1000&2000-HDI-CD (c), L-PEG1000&2000-HDI (d), and magnified TEM image of H-PEG1000&2000-HDI (e).

the size of PEG1000&2000-HDI-CD (H-Mw or L-Mw) was larger than that of PEG1000&2000-HDI. We speculated that the much higher EE and LC (Table. 2) of PEG1000&2000-HDI-CD, bearing mass of drug encapsulated in RMs, result in the lager RMs size. RMs of H-Mw were dispersed well in ethyl oleate, and the size of which was similar to that in DCM. Precipitation was found for RMs of L-Mw polymers in ethyl oleate. Therefore, only RMs of H-Mw polymers were investigated in ethyl oleate. Our previous study has exhibited that PUs exhibited temperature-sensitivity in aqueous solution.[37] We also investigated the temperature responsibility of different RMs from 20 °C to 35 °C in DCM solution (Fig. 4), from 25 °C to 45 °C in ethyl oleate (Fig. 5 & Fig. S1). The size of PEG-HDI-CD and PEG-HDI series decreased from 180-480 nm to 100-280 nm upon increasing temperature. The size of RMs exhibited different temperature dependent behavior from that of normal micelles.[37] As for normal micelles in aqueous phase, the particle size was increased along with the increased temperature.[38] In the process of forming RMs, the equivalents of water are important to form a water pool inside the reverse micelle.[19] RMs in ethyl oleate (Fig. 5) also exhibited the same tendency as those in DCM upon changing temperature. According to the DSC results, most of the polymer will melt due to temperature increase in the range of 20 - 35 °C or 25 - 45 °C. Thus, thermo-responsive size variations of the nanoparticles were attributed to polymer thawing.

The variable temperature ^{1}H NMR of H-PEG1000&2000-HDI-CD, H-PEG1000&2000-HDI PUs were performed in $CDCl_3$, and the results are presented in Fig. 6. All peaks were visible at

Table 2. Encapsulation efficiency (EE) and loading capacity (LC) of RMs

Polyurethanes	LC（%）	EE（%）
H-PEG1000-HDI-CD	42.1	84.2
H-PEG1000-HDI	34.0	68.0
H-PEG1000&2000-HDI-CD	45.0	90.0
H-PEG1000&2000-HDI	26.4	52.8
H-PEG2000-HDI-CD	21.1	42.2
H-PEG2000-HDI	19.1	38.2
L-PEG1000-HDI-CD	20.6	41.2
L-PEG1000-HDI	7.8	15.6
L-PEG1000&2000-HDI-CD	21.0	42.0
L-PEG1000&2000-HDI	8.1	16.1
L-PEG2000-HDI-CD	17.2	34.4
L-PEG2000-HDI	12.2	24.4

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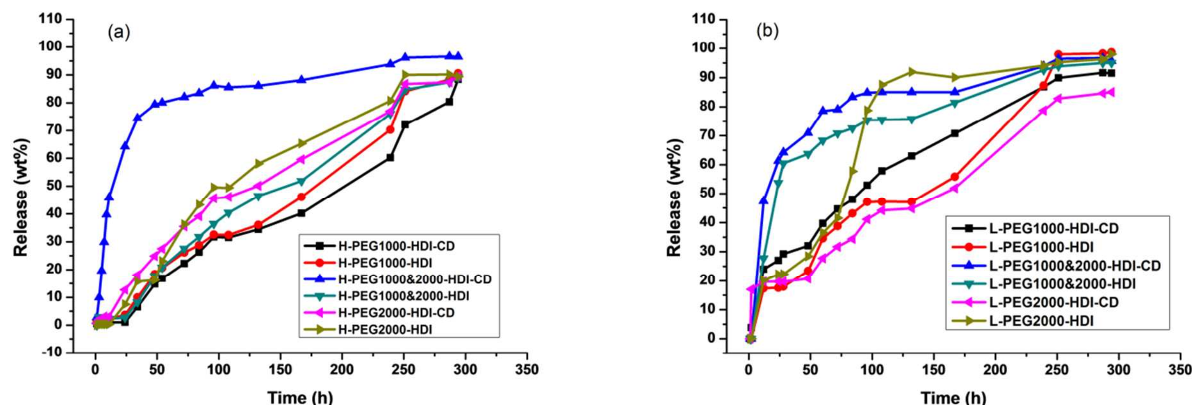

Fig. 8 Transfer of BSA from H-Mw RMs (a) and L-Mw (b) RMs in DCM.

temperatures from 20 to 35 °C. The peak at 2.5 ppm corresponding to the amide (N-H) groups of H-PEG1000&2000-HDI-CD moved to higher field at higher temperature, so did the methylene groups in HDI, which may correspond to the chain re-arrangement upon heating, and explained the size change with temperature variation. The CD-contained PUs displayed more notable change in chemical shift, which was in accordance to the more obvious size variation (Fig. 6a). The hydroxyl groups from CD may enhance the hydrogen-bonding with N-H group, which further influenced its chemical shift in ^{1}H NMR spectrum.[39]

SEM images showed that the RMs at room temperature were nearly spherical in shape (Fig. 7). The mean diameter of H-PEG1000&2000-HDI-CD and H-PEG1000&2000-HDI was about 400 and 240 nm at 25 °C, and that of L-PEG1000&2000-HDI-CD and L-PEG1000&2000-HDI was about 300 nm and 200 nm at 25 °C, respectively, which was similar to that determined by DLS. The size is too large for a typical core-shell structure. Further magnification of TEM evidenced they were nanoparticles with hydrophilic and hydrophobic microdomains coexisting in interior of them.

EE and LC of BSA in the RMs

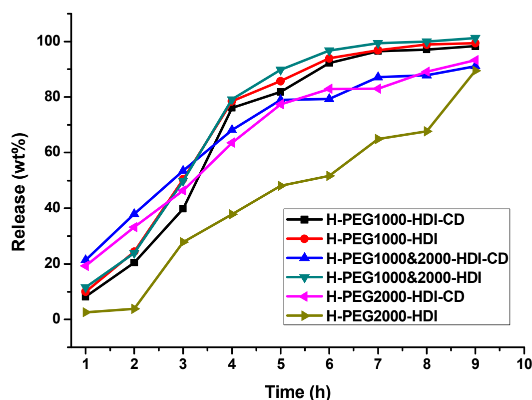

Fig. 9 Release BSA from H-Mw RMs in ethyl oleate.

The EE and LC of BSA in the RMs were calculated from UV orders in the EE and LC of PUs were as following: H-PEG-HDI-CD > H-PEG-HDI, L-PEG-HDI-CD > L-PEG-HDI, H-PEG-HDI-CD > L-PEG-HDI-CD, H-PEG-HDI > L-PEG-HDI,H-PEG1000&2000-HDI-CD > H-PEG1000-HDI-CD > H-PEG2000-HDI-CD, L-PEG1000&2000-HDI-CD > L-PEG1000-HDI-CD > L-PEG2000-HDI-CD. The EE and LC of BSA in the DM-β-CD-contained RMs were much improved in comparison with those without DM-β-CD. EE and LC also increased with DM-β-CD content. H-PEG1000-HDI-CD consistes of 65.6% of DM-β-CD and L-PEG1000-HDI-CD consistes of 20.1% of DM-β-CD possessed the highest EE and LC. DM-β-CD is well soluble in water. For preparation of BSA-encapsulated RMs, BSA was dissolved in water, and then transferred into RMs. Thus, hydrophilic microdomain existed in the RMs, and the inclusion complexes may be formed from the accessible residues of BSA with the hydrophobic cavity of CD moiety, and the cavity size of DM-β-CD was suitable to bind aromatic groups and some large alkyl groups.[40] With the same constituent polymer, EE and LC of BSA in H-Mw PUs were higher than those of L-Mw PUs. The longer chain of H-Mw polymers facilitates the chain coiling, resulting in the enhanced BSA loading. The hydrophilic/hydrophobic balance of the copolymer also influenced the EE and LC. The RMs of composed of shorter PEG hydrophilic chain exhibited enhanced EE and LC. Significantly, the EE of H-PEG1000&2000-HDI-CD and L-PEG1000&2000-HDI-CD were 90% and 42%, respectively, more than the other polymers in H-Mw and L-Mw series. The PEG irregular segment probably effected the arrangement of the molecular chain and improved drug loading.

In vitro BSA release from the RMs

Fig. 8 shows the transfer of BSA from drug-loaded RMs in DCM into water at 25 °C. The transfer rate of protein by diffusion at an early stage was large for the RMs in DCM. The structure of the polymers affected the release of BSA from RMs. The H-Mw PUs can form much more neat construction than L-Mw PUs to accommodate BSA molecules, exhibiting slow

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release rate. A slight increase in the diffusion rate was observed when increasing the molecular mass of the PEG (Fig. 8a). Interestingly, the release rate of H-PEG1000&2000-HDI-CD was larger than other polymers, which could reach up to 85% in 50 h. The irregular PEG segment in alternating copolymer not only increased the loading but also accelerated the release rate of BSA from RMs. The release profile of L-PEG1000&2000-HDI-CD also showed such a trend.

After 10 days, almost all the release reached plateau. The PEG-HDI-CD exhibited faster protein release rate than that of the PEG-HDI. DM-β-CD may provide a channel for BSA diffusion. On the other hand, the size of PU composed of PEG1000 was larger than that of PEG2000, indicating a loose structure of PEG1000-based PUs, which results in a fast release.

The release of BSA from the different RMs in ethyl oleate was studied under 37 °C (Fig. 9). All RMs formulations can release 40-80% of BSA within 4 h, and entered into the extended release phase in 10 h indicating that the release rate could be significantly accelerate after dispersion of RMs in the oleaginous phase, which may inflect the relative loose compact of RMs in ethyl oleate. The influence of structure on release rate is in the similar trend in ethyl oleate as that in DCM.

The CD spectra of the free BSA in the supernatant from the release test after 4 days were measured and shown in Fig. 10. Obviously, two extreme valleys at 205 and 230 nm occurred without any significant difference from those of the native BSA. Compared to the CD spectrum of native BSA, the CD spectrum of the supernatant BSA released from the H-PEG1000-HDI-CD RMs was more consistent than that of BSA released from the H-PEG1000-HDI RMs, indicating that the secondary structure of BSA released from H-PEG1000-HDI-CD RMs was kept more stable than that from the H-PEG1000-HDI RMs.

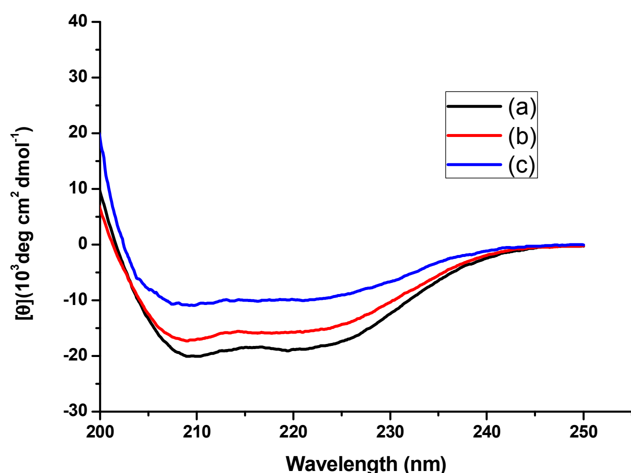

Fig. 10 Circular dichroism spectra of the BSA solutions. (a) Native BSA, (b) BSA release after 4 days from the H-PEG1000-HDI-CD RMs, (c) BSA released after 4 days from the H-PEG1000-HDI RMs.

Conclusions

In this study, H-Mw and L-Mw PUs of PEG-HDI-CD and PEG-HDI were synthesized. All polymers could form RMs in organic/apolar solvent. These RMs showed tunable temperature sensitivity. The size decreased upon heating. In DCM and ethyl oleate, PEG-HDI-CD PUs demonstrated higher protein EE and LC than PEG-HDI PUs. And H-Mw PUs exhibited higher EE and LC than L-Mw PUs. Interestingly, PUs composed of both PEG1000 and PEG2000 showed the highest EE and LC, and fastest BSA release. The stability of BSA in release medium was confirmed by circular dichroism spectra.

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Notes and references

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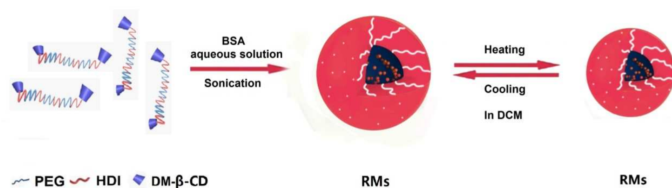
Graphical Abstract

Reverse micelles based on β-cyclodextrin-incorporated amphiphilic polyurethane copolymers for protein delivery

Xiaoxu Du, Nan Song, Ying-Wei Yang, Guolin Wu, Jianbiao Ma, Hui Gao



In DCM and ethyl oleate, all the polyurethanes could form reverse micelles, and PEG-HDI-CD polyurethanes demonstrated higher protein loading than PEG-HDI ones.

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