Electronic supplementary materials *Mendeleev Commun.*, 2020, **30**, 25–27

Hybrid systems for oral delivery of a therapeutic neuropeptide

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Methods

Determination of peptide amount

(1) Spectrophotometric analysis

The absorbance values of peptide solutions at λ=260 nm was measured, and concentrations of solutions were calculated on the basis of the calibration curve (which was plotted using the standard peptide solutions in the same medium).

(2) HPLC analysis

Quantitative analysis was carried out using high-performance liquid chromatography in the ion-exchange mode; the monolithic CIM SO_3 disk (1.2×3 mm) was applied as a stationary phase. The 0.2 M Clark-Labs buffer (pH = 2.0) was used as phase A, and 0.0125 M sodium borate buffer solution (pH = 10.5) was used as phase B. The analysis was carried out according to the following program: 0 - 3 min: 100% phase A, 3.01 - 12 min: 100% phase B, 12.01 - 15 min: 100% phase A. Flow rate was equal to 0.5 mL/min. Injection volume was 20 μL. The U7 retention time was 7.6 min. The amount of peptide was calculated using the peak area value with reference to the calibration curve plotted for U7 standard solutions. This calibration curve represents a dependence of peak area on peptide concentration.

Characterization of particles

Hydrodynamic diameters (D_H) and ζ-potentials of the particles were measured with the use of a Zetasizer ZS (Malvern Instruments, UK) at 25 °C. Scanning electron (SEM) micrographs of the samples of $CaCO_3$ cores and SEC were obtained using a Supra 55VP instrument (Carl Zeiss, Germany). Silica nanoparticles were analyzed by 1) SEM LEO 1550. Poly(amino acid) sub-microparticles were studied with a Jeol JEM-1400 STEM transmission electron microscope (Japan); the samples were placed onto carbonated copper grids and stained with 2% solution of uranyl acetate.

Elemental compositions of the samples were determined by energy-dispersive spectroscopy (EDS) using an X-Max 80 detector (Oxford Instruments, UK).

Determination of drug loading

Loading capacity (L) and loading efficiency (E) were calculated using the following equations:

$$L = (m_i - m_s)/m_P \quad (1)$$

$$E = (m_i - m_s)/m_i \times 100\% \quad (2)$$

where m_i is the initial amount of U7 (mg), m_s is the amount of non-encapsulated U7 in a solution (mg), m_P is the amount of particles (mg).

Release measurements

The following model solutions were used in release studies: (1) simulated gastric fluid (SGF): HCl solution, pH 1.2; (2) simulated intestinal fluid (SIF): 0.1 M sodium phosphate buffer, pH 7.4.

In the case of first level carriers: silica SMPs, $CaCO_3$, SEC particles the selected aliquot was centrifuged and the peptide concentration in the supernatant was measured spectrophotometrically at 260 nm. In the case of poly(amino acid)-based sub-microparticles peptide analysis was carried out by HPLC. For all first level carriers, the determination of the released peptide was carried out by sequentially selecting aliquots from the reaction medium with an appropriate recalculation of concentrations. Aliquots were taken at certain intervals from the suspension of encapsulated particles incubated in SGF or SIF (37°C) under constant stirring. Thus, in all experiments, the data shown in Fig. 2 represent cumulative release.

Preparation and characterization of first-level carriers

1. *Poly(amino acid)-based sub-microparticles*

Amphiphilic copolymer of L-glutamic acid and D-phenylalanine was synthesized *via* ring-opening polymerization of *N*-carboxyanhydrides of γ-benzyl-L-glutamate (Sigma-Aldrich, Germany) and D-phenylalanine (Vecton, Russia) taken in the ratio 4/1 (mol/mol). Weight-average molecular masses (M_w) and polydispersity *(Đ)* of copolymer samples were determined using size exclusion chromatography in DMF containing 0.1 mol/L of LiBr at 40 °C. Flow rate of the mobile phase was 0.3 mL/min. GPC analysis was performed with the aid of a Shimadzu LC-20 Prominence system equipped with a RID 10-A refractometric detector (Kyoto, Japan) and a Styragel Column (dimensions: 7.8×300 mm, HMW6E, bead size: 15–20 μm (Waters, Milford, MS, USA)). The M_w and Đ values were calculated with the use of GPC LC Solutions software (Shimadzu, Kyoto, Japan) and the calibration curve plotted for poly(methyl methacrylate) standards. The copolymer structure was confirmed by ^{1}H NMR spectroscopy (Figure S1), whereas the copolymer composition was established *via* HPLC-MS analysis of amino acids obtained after total hydrolysis of the copolymer [1] (Table S1).

The obtained copolymer possessed amphiphilic properties and tended to self-assemble in aqueous media. Formation of nanoparticles was carried out using the approach that involves gradient phase inversion (dialysis) from the organic medium (DMF) to water.

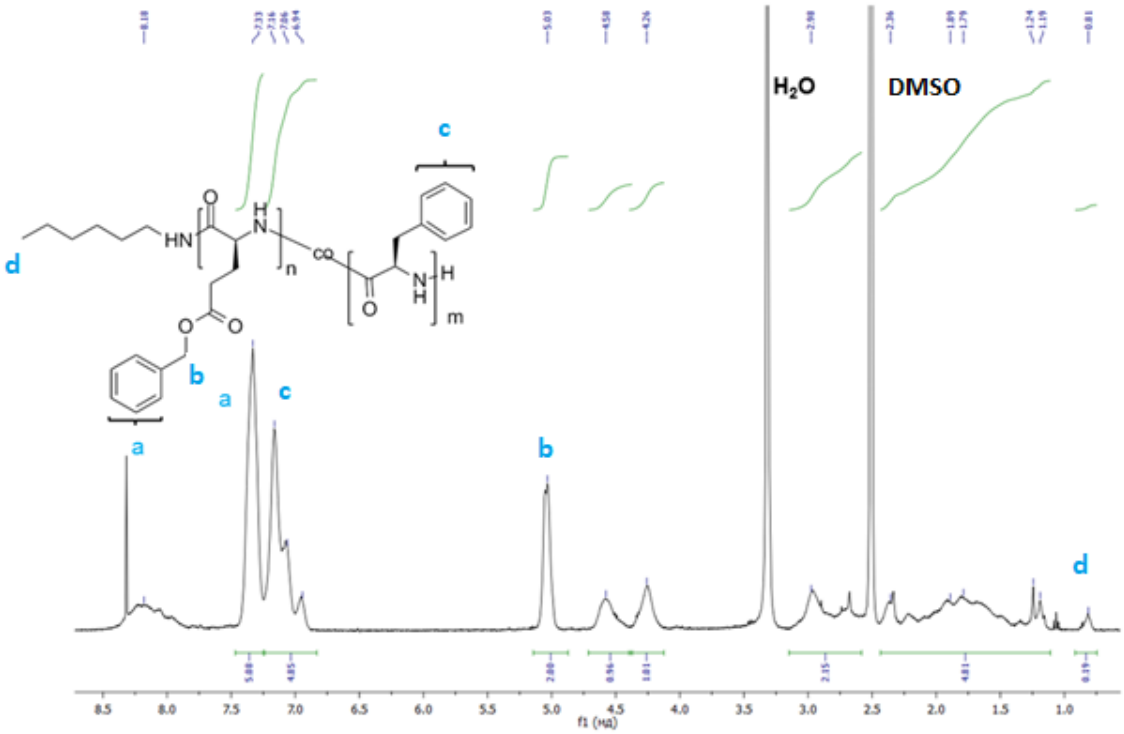


Figure S1 ^{1}H NMR spectrum of poly(LGlu(OBzl)-*co*-DPhe) in DMSO-d_6.

Table S1 Composition of poly(LGlu$_n$-*co*-DPhe$_m$)

n (Glu)	m (Phe)	Amino acid content/ mol%	
		LGlu	DPhe
30	11	71	29

Encapsulation of U7 peptide was performed *via* intensive stirring of the peptide solution containing suspension of particles in 0.1 M Na-phosphate buffer (pH 7.4) for 30 min at 22°C. Then the mixture was incubated overnight at 4 °C. 0.5 mg of U7 peptide was taken per 1.0 mg of the copolymer. Free peptide was removed from the suspension via ultrafiltration through the membrane columns with MWCO 3500.

2. Silica SMPs

Synthesis of silica sub-microparticles (SMPs) was performed according to the following procedure. Tetraethoxysilane (TEOS, 4 g) and water (1.668 g) in the 1:4 molar ratio were placed in a flask equipped with a magnetic stirrer. Then a small amount of 5% solution of ammonia was added dropwise to the system in order to perform alkaline hydrolysis (pH 8.0). Thereafter, 1 mL of U7 peptide solution (35 mg / mL) was added. The synthesis was carried out at constant stirring for 2–3 h, after which stirring was stopped, and the system was left to stand for two days until the stage of hydrolysis and polycondensation of TEOS was completed. The resulting material was filtered off and dried under vacuum at 95 °C until a constant weight was reached.

3. $CaCO_3$ microparticles

Porous vaterites - $CaCO_3$ microparticles (MPs) were formed as a result of precipitation during the reaction between calcium chloride and sodium carbonate. We used the technique described by the authors of [2] and introduced some modifications into the washing procedure.[3]

Briefly, a certain volume of water solution of $CaCl_2$ (C=1 M) was rapidly added to water solution of Na_2CO_3 of the same volume and concentration during mixing with a mechanical stirrer (1000 rpm). The mixture was stirred for 30 s, and then the obtained suspension was left for 1 min.

For doping of $CaCO_3$ MP polyanion dextran sulfate with MM = 9-20 kDa supplied by Sigma Aldrich was used. The doping of was performed according to the developed procedures.[3]

During the formation of $CaCO_3$ MPs doped with DexS by coprecipitation, the DexS was dissolved in Na_2CO_3 at a concentration 0.6M. The subsequent procedure was similar to that described above.

Doping of $CaCO_3$ MPs with DexS by covering was performed as follows. Aqueous solution of the polymer (C = 0.6 mg/mL) was added to the pre-made particles. Then the suspension was stirred using a Multi Bio RS-24 rotator (Biosan, Latvia) for 30 min, and centrifuged for 3 min at 3000 rpm. Supernatant was removed and substituted for water in order to wash cores from free polymer. The washing procedure was performed twice.

In all three cases, washing and drying ~~of~~ were performed with the aid of Schott filter glasses (#16); the MPs were washed using acetone/water mixtures with increasing acetone concentrations (up to 100%). The final drying of MPs was performed in thermostat at 30-50°C. The values of ζ-potential and content of S atoms in the pristine and doped $CaCO_3$ MPs are presented in Table S2.

Table S2 Characteristics of the pristine $CaCO_3$ and the microparticles doped with DexS.

Sample	ζ-potential/ mV	Content of S atoms (%)
Pristine $CaCO_3$ MPs	-24.9	0
$CaCo_3$ MPs co-precipitated with DexS	-26.1	1.2±0.3
$CaCO_3$ MPs covered by DexS	-29.0	0.8±0.3

4. Sporopollenin exine capsules (SEC), or Lycopodium clavatum spore shells

SEC were prepared by removal of the pores' content as described in.[4,5] The following procedure was used: a weighed amount of spores (25 g) was suspended in 75 mL of acetone at stirring for 4 h, and then dried in air. The resulting product was suspended in 75 mL of 6% solution of potassium hydroxide and stirred for 6 h. After filtration, the procedure was repeated. Then the spores were filtered off, washed with hot water and hot ethanol, dried in air and suspended in 75 mL of 85% orthophosphoric acid at stirring for 7 days. Then the spores were filtered off, washed with water, acetone, 2 M HCl solution, 2 M NaOH solution, water (until neutral pH was achieved), acetone, and ethanol, and dried at 60°C. SEC samples were intensively washed with distilled water under the control of the structure stability before drying.[5] Washing was performed 4–6 times.

Silanization of SEC was performed as follows: 1 g of SEC was placed into a glass weighing bottle, acetone (15 mL) was poured, the lid was closed, and the mixture was stirred using a magnetic stirrer. After 5 minutes, 0.1 mL of tetraethoxysilane (TEOS) was added; after 10 minutes, 0.2 mL of water was added, and the mixture was stirred for 1 hour. The suspension was then filtered through Schott glass filter (#16), washed with acetone and dried in a dry box until the smell of acetone disappeared.

The presence of SiO_2 coating on the surface was confirmed by determining atomic composition of the surface by EDS. Comparison of surface atomic compositions of the pristine and modified SEC is given in Table S3. (The average error calculated on the basis of 6-10 measurements is 5-15%).

Table S3 Atomic compositions of the surface SEC/ weight %.

Sample	C	N	O	S	Na	Si	Ca
Pristine SEC	88.19	0.89	10.44	0.43	-	-	-
Silanized SEC	77.88	0.97	19.93	0.11	0.63	0.08	0.39

Preparation of two level delivery systems

Two level delivery systems (DS) were prepared according to the following technique. To obtain two level DS, first level carriers were dispersed in 3% solution of sodium alginate. Suspension of first level carriers in solution of sodium alginate was sprayed into gelation bath (GB) through the spinneret consisting of two tubes; diameter of the outer tube was 2 mm, and diameter of the inner tube was 0.8 mm. The solution was fed through the internal tube, and compressed air was fed through the external tube. The size of obtained alginate granules (AGs) was controlled by the pressure of compressed air. In all cases, GB contained 1% solution of $CaCl_2$ in 0.5% solution of chitosan (which, in turn, was dissolved in 1% solution of acetic acid). AGs were exposed to GB for not more than 10 min. Then, AGs were filtered off, washed with water and dried in air for 24 hrs.

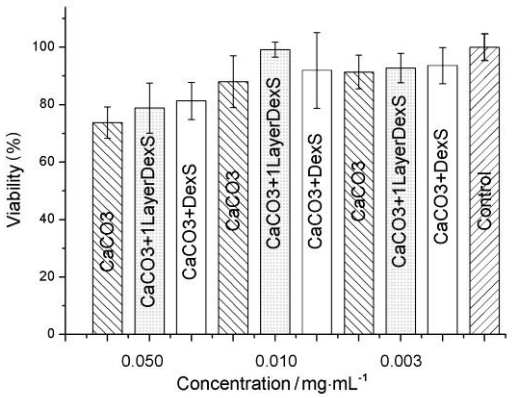
Sodium alginate intended for medical use (with molecular weight of about 300000) was purchased from Arkhangelsk Experimental Seaweed Production Plant (Arkhangelsk, Russia). Chitosan of medium viscosity (molecular weight about 220000) was purchased from Sigma Aldrich.

Loading of peptides into the pristine and modified silicate SMPs, $CaCO_3$ cores and SEC, as well as the kinetics of their release from the first level carriers and from the two level SD. into the simulated media, were controlled by spectrophotometric determination of peptide concentrations at λ=260 nm. The linear calibration dependences in the respective media were used. The methods for determination of peptide loading and release were described above in more detail. The release of the peptide from two level systems was controlled using the Lowry method[6] instead of determining the absorption of the peptide solution at 260 nm. All experiments were carried out thrice.

Cell viability assay

Before contact with cells, the first level carriers of all types used were sterilized for 40 min at 120°C. The MTT viability assay was performed for the assessment of cell growth. For the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) assay, A431 cells were seeded into wells of the 96-well plate (each containing 200 μL of culture medium) at a density of $5x10^3$ cells / well. Cells were treated with different concentrations of the pristine and doped cores for 5 days. MTT (Sigma Aldrich, USA) was supplied as a stock solution (5 mg/mL culture medium) and sterile-filtered. At the end of the treatment period, MTT solution was added to all the wells, and incubation was performed for 2 h at 37^{o}C. 100 μL of DMSO was then added to each well for 5 min incubation at 37 ^{o}C in thermo-shaker. The absorbance values were determined with a SpectroSTAR Nano plate reader at 570 nm (BMG Labtech, Germany). The absorption value is proportional to the amount of cell viability (growth).

(a)



(b)

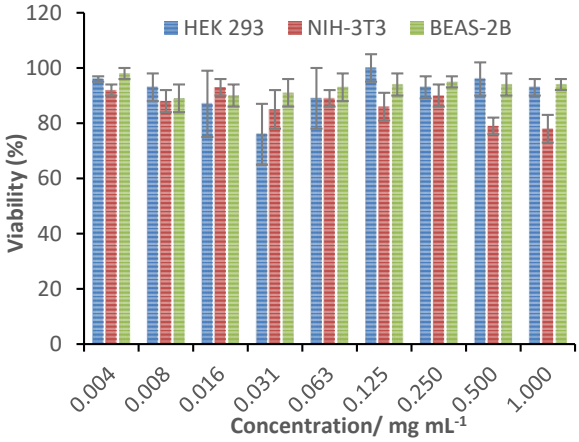


Figure S2 Viability results obtained for different concentrations of pristine and DexS doped $CaCO_3$ cores (a) and poly(LGlu-*co*-DPhe) (b) nanoparticles.

References

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