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Hybrid systems for oral delivery of a therapeutic neuropeptide

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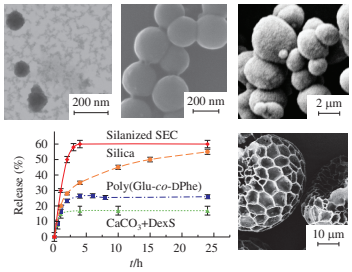
Several hybrid systems for oral delivery of a therapeutic peptide have been prepared and investigated. Poly(L-glutamic acid-*co*-D-phenylalanine) and silica sub-microparticles, porous $CaCO_3$ microparticles and *Lycopodium clavatum* spore capsules were employed as the first level carriers included in sodium alginate granules as the second level carriers. Efficiency of the peptide encapsulation and release rate strongly depended on the nature and structure of the carriers.



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Peptide drugs have proven to be highly efficient because of their specificity and minimal side effects.[1,2] Due to the latter property, the peptide pharmaceuticals are suitable for long term use. When they are prescribed many a time, it is appropriate to replace parenteral administration by an oral one, given the bioavailability is comparable. It is known that after oral administration, the peptide drugs degrade quickly due to enzymatic and acidic hydrolysis in human gastrointestinal tract. To overcome this drawback, an application for the oral peptides delivery using polymer-based or inorganic micro- and sub-microparticles (SMPs) can be considered as a suitable approach. The related parameters of loading efficiency *E* and drug loading *L* as well as drug release profile are known to depend on the peptide nature and characteristics of a carrier, namely the size of particles and their pores, surface morphology and ζ-potential, as well as on the encapsulation methods applied, such as entrapment, coprecipitation and passive or active sorption.

In general, oral administration of therapeutic peptides is followed by their penetration into blood circulation either after release from intestinal mucosa, or as a result of liberation from the carriers that have already entered the blood. Various ways of penetration across the intestinal barrier for the particles of different sizes are described by the corresponding models.[3] As a rule, the smaller the compound piece, the higher the probability of its penetration into the blood. Nevertheless, the 25 μm sized spore capsules, also called as sporopollenin exine capsules (SECs), were found in the rat[4] and human[5] blood after oral administration.

To protect the peptide drugs in the gastrointestinal tract after the oral administration, various delivery approaches have been designed, in particular the hybrid systems, which represent alginate granules (AGs) as the second level carrier containing the first level carrier loaded in turn with the peptide.[6] It is known that alginate is a pH-sensitive polymer,[7] and the polymer coil contracts in the acidic environment of stomach, thus protecting its content from enzymatic and acidic destruction. Later, after entering the intestinal tract with its alkaline environment, the AGs swell and release the first level carrier accompanied by the peptide. Additional introduction of inhibitors of peptidase and/or trypsin into the AGs helps to protect their content against intestinal enzymatic degradation.[5,6] The first level carriers can be of various nature, including inorganic particles like gold nanoclusters[8] or metal oxides,[9] $CaCO_3$ porous particles,[10,11] silica[12] or polymeric SMPs,[13–15] microemulsions[16] as well as products from natural sources, for example spore capsules.[17]

In this work, we compared several hybrid systems for delivery of therapeutic neuropeptide KSQTPLVTLFK (U7), which represents β-endorphin fragment 9–19. The selected peptide has a molecular weight of 1261 Da and p*I* = 9.4, that is, it carries positive charge in a neutral environment. The two level hybrid systems were prepared from the first level carriers of different nature, structure and size, namely the poly(amino acid) and silica SMPs as well as microparticles based on porous $CaCO_3$ and SECs, whereas sodium alginate was used for the preparation of pH-sensitive granules as the second level carriers. The selected materials are known to be biocompatible[10,12] and do not show cytotoxicity.[3,6,18] A detailed description of the first level carriers used in this work is given below.

Poly(amino acids) as biodegradable polymers are known to be used in the preparation of delivery systems (DSs) for various hydrophobic and hydrophilic drugs.[19] To encapsulate the chosen peptide, self-assembled SMPs based on the random copolymer of L-glutamic acid and D-phenylalanine were prepared, the latter amino acid was selected to slow down the enzymatic degradation of

the polypeptide SMPs. The copolymer was synthesized *via* ring-opening polymerization of the corresponding amino acid *N*-carboxy-anhydrides in 4 : 1 molar ratio. According to the analysis by size exclusion chromatography in DMF with a refractometric detector, the copolymer had $M_w = 8100$, $M_n = 6700$ and $Đ = 1.20$, as calculated from the calibration dependence plotted using poly(methyl-methacrylate) standards. The SMPs were formed *via* self-assembly of the amphiphilic copolymer in a gradient phase inversion from the organic DMF medium to the aqueous one, namely 0.01 M sodium phosphate buffer (pH 7.4).

Silica SMPs were prepared *via* sol–gel technique using tetra-ethoxysilane as a precursor, whose hydrolysis in a basic aqueous medium led to the formation of Si–OH bonds.

Porous $CaCO_3$ particles were obtained by mixing equal volumes of equimolar Na_2CO_3 and $CaCl_2$ solutions under stirring, followed by $CaCO_3$ nucleation and growth. Doping of the $CaCO_3$ particles was performed in two ways: (i) by coprecipitation in the presence of dextran sulfate (DexS) polyanion during nucleation or (ii) by coating of the particles surface by the DexS polyanion during their immersion in the polymer solution. The size of the particles formed by the first way was smaller than for ones with a surface coated by DexS.

Lycopodium clavatum SECs were obtained by removing content of the spores *via* sequential extractions with acetone, alkaline and acidic solutions, ethanol and finally with water. The details of the preparation and characterization of all the first level carriers can be found in Online Supplementary Materials.

Figure 1 illustrates the morphology of the first level carriers visualized by electron microscopy. Contrary to other considered DS that were rigid, the poly(LGlu-*co*-DPhe) SMPs were soft ones and were formed due to the polymer self-assembly. The morphology of poly(LGlu-*co*-DPhe) SMPs was evaluated using transmission electron microscopy (TEM), which involved staining with uranyl acetate. The obtained pattern [Figure 1(*a*)] is typical of polymer micelles with hydrophobic core and hydrophilic surface.[20] According to TEM, the size of poly(LGlu-*co*-DPhe) SMPs was about 0.1 μm, which was two times lower than the value of hydrodynamic diameter determined by DLS (Table 1). This difference is known for soft self-assembled materials[21,22] and originated from deflation of water upon drying on the TEM grids.

To visualize the morphology of other particles, scanning electron microscopy (SEM) was used [Figure 1(*b*)–(*e*)]. Both silica and $CaCO_3$ particles were spherical, the surface of silica SMPs was smooth, whereas the $CaCO_3$ microparticles had a porous surface. In turn, SEC microparticles had a honeycomb structure, whose surface was pierced with nanometer-sized pores.[5]

The structural and functional characteristics of the first level and the two level materials are summarized in Table 1. The difference in

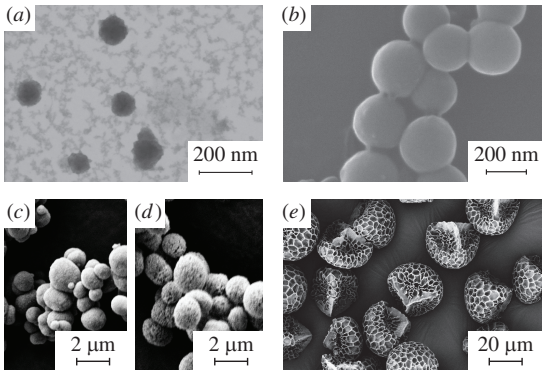

Figure 1 (*a*) TEM image of poly(LGlu-*co*-DPhe) SMPs. SEM images of (*b*) silica SMPs, (*c*) $CaCO_3$ microparticles coprecipitated with DexS, (*d*) $CaCO_3$ microparticles coated by DexS and (*e*) SEC microparticles.

nature, morphology, rigidity and preparation methods of the first level materials under investigation required various techniques for the peptide loading. The methods of manufacturing, processing and characterization of the DSs are described in detail in Online Supplementary Materials.

For the poly(amino acid) SMPs, encapsulation was carried out *via* entrapment of the peptide during the polymer self-assembly. The maximum loading value for the peptide was ~380 μg mg^{-1}, which corresponded to loading efficiency *E* of 76%. To increase mucoadhesive properties of the polymer system, the poly(LGlu-*co*-DPhe) SMPs loaded with U7 peptide were additionally covered with chitosan. The optimal SMPs to chitosan ratio was 5 : 1. On the one hand, the coating of SMPs achieved at this ratio resulted in the change in ζ-potential from –45 to +12 mV, on the other hand, the hydrodynamic diameter D_H of SMPs did not increase so drastically as in the case of lower ratio of SMPs to chitosan. After the coating of SMPs, their D_H value increased from 0.21 ± 0.01 to 0.32 ± 0.07 μm.

Encapsulation of the peptide into silica SMPs was also carried out *via* entrapment in the course of their preparation. Similar to the previous case, this approach allowed us to achieve high values of *L* and *E* (see Table 1). No additional surface modification was performed for this DS.

For the $CaCO_3$ particles, the method involving sorption under centrifugation had been earlier found as the most efficient technique for peptide loading.[6] The attempt to load the peptide during coprecipitation resulted in very low loading values, moreover, the loaded peptide was partially removed from the particle pores at the washing step. In this work, we doped the $CaCO_3$ microspheres with DexS, and as a result the peptide loading increased several times (see Table 1) due to electrostatic

Table 1 Peptide oral delivery systems developed.

1st Level carrier	Treatment of carrier	Size/μm	ζ-Potential/ mV	Loading method	*L*/μg mg^{-1}	*E* (%)	Release from 1st level carrier in SIF[a] after 24 h (%)[b]	Release from 2-level DSs (%)[b]	
								In SGF[c] after 2 h	In SIF after 24 h
Poly(LGlu-*co*-DPhe) SMPs	Chitosan	0.20–0.30	12	Entrapment	380 ± 10	76	26	–	16
Silica SMPs	–	0.10–0.25	–38	Entrapment	500 ± 30	49	55	5	45
$CaCO_3$	–	1–2	–24	Sorption under centrifugation	15 ± 5	3	37	10	30
$CaCO_3$	Coprecipitation with DexS	0.3–1.5	–26	Sorption under centrifugation	70 ± 8	10	17	3	18
$CaCO_3$	Covering by DexS	1–2	–30	Sorption under centrifugation	90 ± 8	23	7	2	10
SEC	–	25	–	Sorption *in vacuo*	45 ± 15	100	70	45	58
SEC	Silanization	25	–	Sorption *in vacuo*	45 ± 15	100	60	25	40

[a] SIF is simulated intestinal fluid. [b] The standard errors in determination of these values are 3–7%. [c] SGF is simulated gastric fluid.

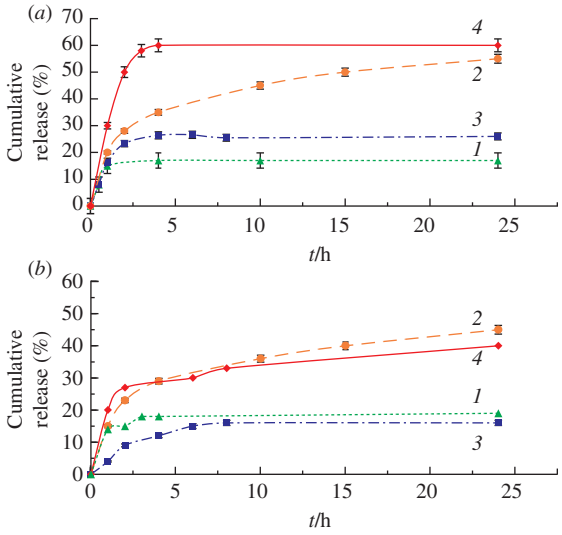

Figure 2 Time dependence for U7 peptide release in SIF at 37 °C (*a*) from the first level carriers and (*b*) from these carriers entrapped into alginate gel: (*1*) $CaCO_3$ particles covered with DexS *via* coprecipitation, (*2*) silica SMPs, (*3*) poly(LGlu-*co*-DPhe) SMPs and (*4*) silanized SEC particles.

interaction between the negatively charged surface of particles and the positively charged peptide. The apparent advantages of $CaCO_3$ as a carrier are based on its low cost and simplicity of preparation.

For the SEC microparticles, peptide loading was carried out by vacuum sorption, *viz.* the microparticles were put into the peptide solution and then the mixture was dried *in vacuo*. This technique facilitated total adsorption of the introduced peptide on the surface of SEC carrier. To lower the peptide leakage under acidic conditions, the surface of SECs was additionally silanized by tetraethoxysilane (see Online Supplementary Materials).

All the first level carriers loaded with U7 peptide were further entrapped into AGs during the granules formation *via* ionotropic gelation of the first level carrier suspension in sodium alginate in the presence of $CaCl_2$ and chitosan. The size of the irregular shaped spheres of AGs prepared after that by the spraying method did not exceed 200 μm.

The investigation of the U7 peptide release profiles for the obtained DS was carried out in protease-free simulated intestinal fluid (SIF) as well as in simulated gastric fluid (SGF). The comparison of the release profiles from the first level carriers in SIF for 24 h [Figure 2(*a*)] revealed that the highest release values were achieved with native and modified SECs, while the lowest ones were observed for poly(LGlu-*co*-DPhe) and doped $CaCO_3$ particles. The data in Table 1 demonstrate the change in the release values in going from the first level carriers to the two level systems. The structure of AGs creates additional resistance to mass transfer, which reduces the peptide release. Similar effect was observed after silanization of SECs.

Cytotoxicity is an important characteristic of the investigated DSs, because the accumulation of SMPs in a body after their administration can result in a negative effect. Alginate is a component of all the investigated systems, and it is known that this biocompatible and biodegradable polymer is used in various fields of medicine and does not reveal toxicity.[3] SiO_2 and $CaCO_3$ SMPs as well as SEC particles are also known to be nontoxic.[5,23,24] The cytotoxicity of systems, based on parent and doped $CaCO_3$ as well as poly(LGlu-*co*-DPhe) SMPs in the concentration range corresponding to a therapeutic dose of the peptide, was tested by MTT assay and proved to be negligible (see Online Supplementary Materials). Moreover, doping of the $CaCO_3$ particles with DexS further reduced their toxicity.

In summary, structural modification of carriers led to the improvement in the functional properties of hybrid delivery systems. In particular, the systems with lower size and additionally modified surface demonstrated higher peptide loading, along with the known considerable increase in the ability to penetrate cell membranes with a decrease in the carrier size.[25] Furthermore, after definite treatment of some carriers, negligible or no release was observed in SGF after 2h. Each of the investigated hybrid systems has a number of apparent advantages. Taking into account their structural and *in vitro* functional characteristics, we can recommend the two level alginate granules with poly(LGlu-*co*-DPhe) and the SiO_2 SMPs as promising first level carriers for the oral delivery of therapeutic peptides.

Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.mencom.2020.01.008.

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