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PREPARATION AND CHARACTERIZATION OF MICROCAPSULES BASED ON PHOSPHORYLATED CURDLAN AND HYDROLYZED COLLAGEN

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Hollow microcapsules composed from a natural polyelectrolyte and a protein were obtained using the layer-by-layer adsorption on silica oxide particles as template. For the first time, monobasic curdlan phosphate (PCurd) is used as anionic partner in self-assembled multilayers fabrication. The deposition of (curdlan phosphate /hydrolyzed collagen) multilayers was monitorized by ζ - potential measurements. The microparticles morphology assembled with four double-layers were investigated by optical and electron microscopy techniques, and atomic force microscopy. The hollow microcapsules obtained by the removal of the colloidal template with hydrofluoric acid solution were characterized by SEM-EADX and termooxidative analyses.

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1. Introduction

In the last decade, the technique of self-assembly layer-by-layer (LbL) has been applied in various micro-encapsulations [1-3]. This method, based on the electrostatic interaction between oppositely charged polyelectrolytes, was first introduced by Decher et al. to fabricate thin films on planar supports by alternate adsorption of polycation and polyanion [4]. Due to its advantages of fine control of the compositions and the thickness of layers, the LbL method has drawn more attention in the recent years [3, 5]. It has been extended to three-dimensional systems by Mohwald et al. to fabricate core-shell particles and hollow capsules by further removal of the template core [6]. The fabrication of micro- and nano-capsules which enable the encapsulation of various materials is of interest in pharmaceutics, medicine, food industry, cosmetics, and agriculture [1, 7]. The removal of the encapsulated template is a key step in capsule fabrication. Several templates have been used for the capsule preparation such as organic polymers: melamine-formaldehyde [3, 8-10], polystyrene [11] or inorganic compounds: silica particles [12-15], $CaCO_3$ [16, 17], $MnCO_3$ [18, 19], iron oxide [20], and gold or silver particles [21]. Using this approach, some natural polyelectrolytes based on polysaccharides have attracted attention in the last years, due to their biocompatibility, biodegradability and being renewable materials. Recently, dextran sulfate [10, 13, 22], chitosan/chitosane sulfate [8, 13, 22, 23], sodium alginate [8, 16], carboximethylcellulose [16], curdlan derivatives [12, 24] have been reported as being used in the obtaining of nano- / micro capsules.

In order to obtain microcapsules as carrier for active principles (e.g. drugs, enzymes, vitamins), the build-up of ordered molecular assemblies based on LbL technique using a natural polyelectrolyte and a polypeptide was here realized. The curdlan phosphate (PCurd) was used for the first time as a natural anionic partner. PCurd is a derivative of curdlan with monobasic phosphate groups, soluble in water, obtained in our laboratory by the reaction of curdlan with

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phopsphorous acid [25]. Curdlan is a bacterial polysaccharide with a linear structure, formed by pure culture fermentation of *Agrobacterium biobar 1*. It is composed entirely from D-glucose units linked by β-(1→3) glucosidic bonds. As reported previously, curdlan has a potential inhibitory effect against AIDS virus infection, blood anti-coagulant activity, anti-oxidant or anti-tumour activity, immunomodulatory effects [24, 26-30].

Hydrolyzed collagens (HC) are natural proteins derived from the collagen by neutral or acidic hydrolysis. Collagen represents the most abundant structural protein from the vertebrate body. The collagen macromolecule consists of three polypeptide chains twined around one another and the hydrogen bonds assure the stabilization of the triple-helix [31, 32]. The neutral, acid or enzymatic hydrolysis of collagen leads to the breaking of the spiral native configuration. These treatments lead to soluble products (HC) that adopt a coiled configuration in solution.

In this paper, the synthesis of the microcapsules by layer-by-layer technique of PCurd and HC was presented. The silica particles were used as template in LbL deposition. The LbL deposition was monitored using ζ-potential measurements. The microparticles / hollow capsules have been characterized by optical microscopy, scanning electronic microscopy (SEM-EDAX), atomic force microscopy (AFM), and termooxidative analyses. These microcapsules can be used for drug encapsulation with pharmaceutical applications.

2. Experimental

2.1. Materials

The mesoporous silica (MS) type Daisogel (Daiso Chemical Co., LTD., Japan) was used as inorganic substrate material. The mean diameter of the microporous silica particles was 50 μm and the distribution of the pore diameters has a maximum of 100 nm. The monobasic curdlan phosphate (PCurd, with a substitution degree of about 1) was synthesised in our laboratory [25]. The hydrolyzed collagen was purchased from National Research and Development Institute for Textile and Leather, Bucharest, Romania (M_v = 105000) [33]. The advanced purification of both partners was realized by diafiltration and the polymers were recovered from the solution by freeze-drying. Sodium hydroxide, hydrofluoric acid and sodium chloride (purchased from Sigma Aldrich) were used as received. All experiments were performed using twice-distilled water.

2.2. Methods

2.2.1. Preparation of microcapsules through layer-by-layer assembly

The hollow capsules were fabricated by alternating immersion of eight layers HC/PCurd onto MS using the LbL technique (Fig. 1). First, the negatively MS were immersed in an aqueous solution of HC (1.0 mg/mL, 0.1M NaCl) with pH=4 for 15 min at ambient temperature and occasional shaking. The pH of HC solution was maintained between 3.5-4 because at this pH the number of positive charges is greater than the number of negative charges [32]. To facilitate the transport into the mesopores of the protein, the absorption of the first layer was conducted under sonication for 15 min. The excess of HC was removed by washing with twice distilled water with the pH adjusted to 4. The next coating step was carried out, with an aqueous solution of negatively charged polyelectrolyte (PCurd, 1.0 mg/mL, 0.1M NaCl) in the same manner. The procedure was repeated for eight times. The pH value of polysaccharide and polypeptide solution was adjusted to 4 with 0.1M HCl or NaOH. The hollow capsules were obtained by dissolving the coated particles in 0.3 M HF at 4°C for 48h and then washed with twice distilled water.

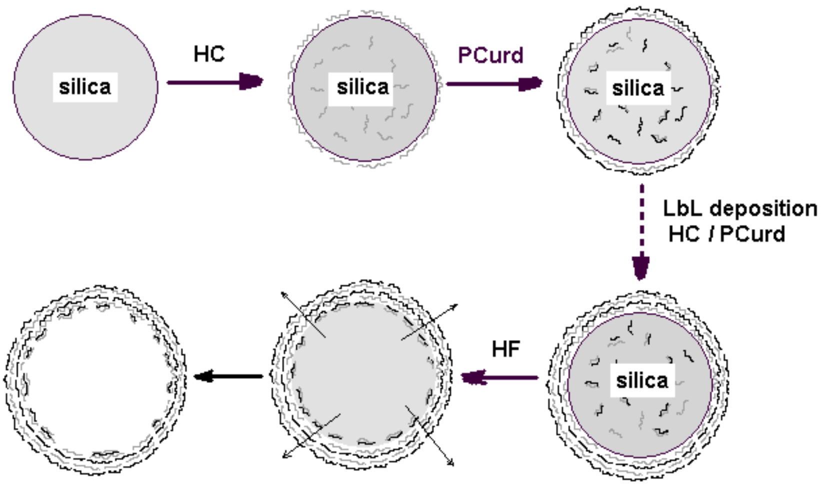


Fig. 1. Consecutive adsorption of hydrolyzed collagen and monobasic curdlan phosphate onto negatively charged silica particles. After dissolution of the silica core in HF solution, the hollow capsules were obtained.

2.2.2. Characterisation of micro-particles/capsules

Streaming potential measurements were carried out to determine the zeta (ζ) potential of polysaccharide/polypeptide multi-layers. Because the average diameter of the silica particles was 50 μm, the ζ-potential measurements could not be performed with Zetasizer. That is why the electrokinetic properties were determined using a Surpass electrokinetic analyzer from Anton Paar GmbH. The zeta potential was estimated at the interface between the modified silica particles/electrolyte based on the measurement of streaming potential and streaming current. For each experimental determination modified-silica particles were placed in a cylindrical cell limited by two AgCl electrode through an AP Nitex 03-50/31 filter type, with a pore size up to 25μm. Approx. 0.2 g of unmodified and modified-silica particles were introduced into the cylindrical cell of the electrokinetic analyzer. The zeta potential in twice-distilled water was determined after the sample was rinsed for 300 sec. Also, the change of the specific electrical conductivity of the measurement solution was examined. The ζ-potential value was calculated using the Fairbrother-Mastin relation, with the effect of the surface conductivity (Equation 1):

$$\zeta = \frac{dU}{dp} \times \frac{\eta}{\varepsilon \times \varepsilon_0} \times K \qquad (1)$$

where U is the streaming potential, p is the pressure, ε_0 is the vacuum permittivity, and η and ε are respectively the viscosity and dielectric constant of the electrolyte solution and K is the electrolyte bulk conductivity. The streaming potential coefficient dU/dp is obtained by a linear regression of the continuous increase in streaming potential with the differential pressure across the powder sample.

The *optical microscop*e images were obtained with a Leica Microscope. The silica particles, silica with $(HC/PCurd)_2$, and $(HC/PCurd)_4$ were deposited on the glass slide. The images were taken at the same light intensity and size.

Scanning electron microscopy (SEM- EADX) observation was performed using a Quanta 200 (FEI) electron microscope equipped with EDX system.

The *atomic force microscopy* measurements were made on a Scanning Probe Microscope Solver Pro-M platform (NT-MDT, Russia), in air, at room temperature (23°C), in a tapping mode. A rectangular silicon cantilever NSG 03 (NT-MDT, Russia), with a typical force constant K_N = 0.35-6.06 N m^{-1} and 47-150 kHz oscillation frequency was used. The tip curvature radius and height were 10 nm and 14-16 μm, respectively. The scan area was 2x2 μm^2, 256 x 256 scan point size images being thus obtained. For image acquisition, analysis and the calculation of the surface texture parameters, the last version of the NT-MDT NOVA software was used.

The *particles size distribution* was measured by laser diffraction method using a Malven Mastersizer 2000 instrument.

The decomposition under dynamic conditions of heating has been investigated with a Paulik-Paulik-Erdey MOM-Budapest instrument on 50 mg samples, at 12°C/min, in air.

3. Results and discussion

The stepwise growth of multilayer shells onto MS was monitored by measuring the ζ – potential charges for the particles after each deposition step (Fig. 2). These alternate changes in the ζ-potential, after each new HC and PCurd adsorption, are characteristic of the LbL formation.

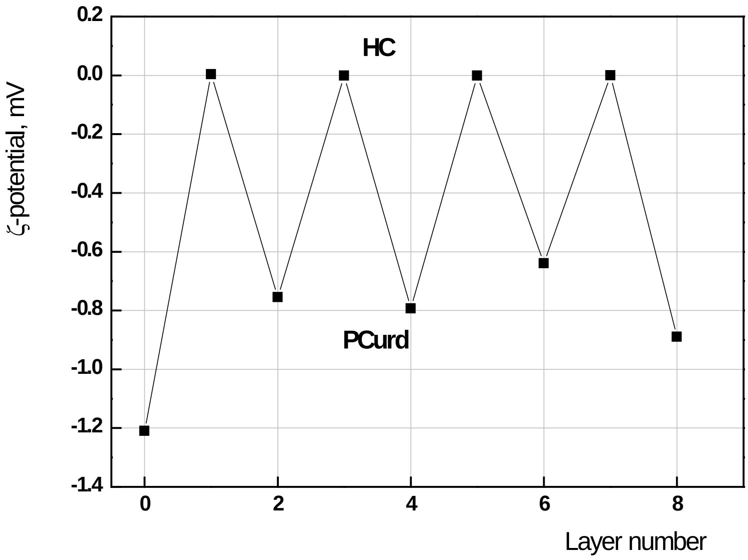


Fig. 2. Variation of the ζ-potential after the deposition of HC/PCurd multilayer shells on MS microparticles. The values are the mean of three independent experiments that deviated with 0-5%

The ζ-potentials of the silica particles with 1 layer of HC shifted from +0.015mV to -0.755mV when PCurd was deposited. The positive values of the ζ-potential are very low, probably due to the amphoyte structure of the hydrolyzed collagen: both positive and negative charges are present on the collagen chain and the ζ-potential of the HC solution is positive but relatively small at pH=4 [33].

The build-up of the HC/PCurd multilayer on MS microparticles was also investigated using an optical microscope. Figure 3 shows the growth of the number of layers observed by optical microscopy at the same magnification and at the same light intensity.

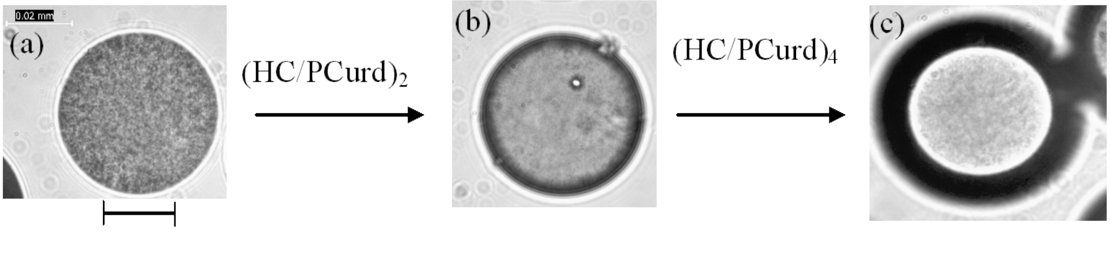
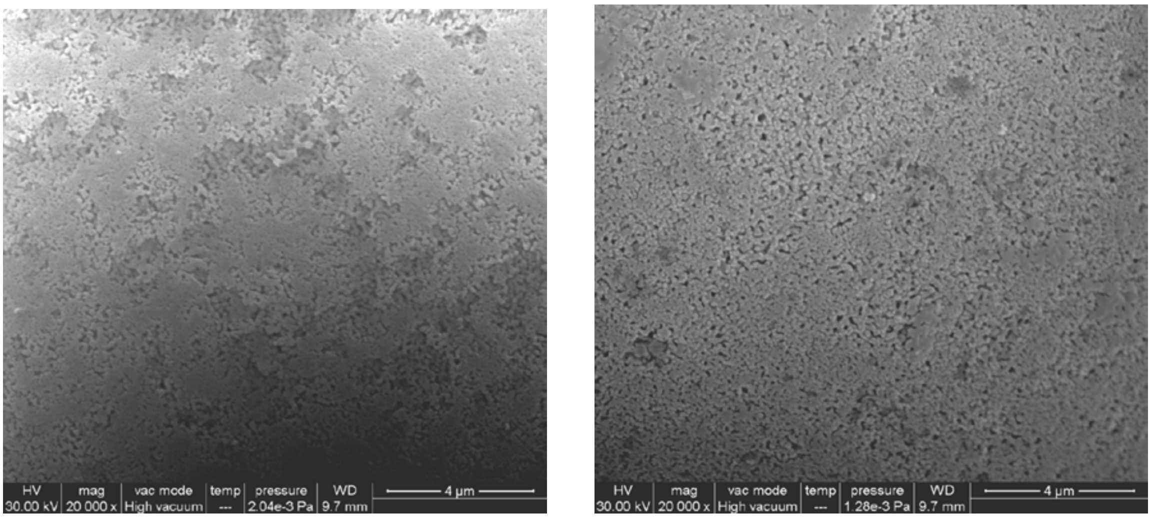


Figure 3. Optical images of silica particles (a), silica with (HC/PCurd)$_2$ layers (b), and silica with (HC/PCurd)$_4$ layers (c). The bar corresponds to 20 μm.

The adsorption of HC/PCurd layers on MS was also studied by SEM (Fig. 4). A change of the surface smoothness of the particles after deposition of HC/PCurd was observed. Also, the presence of polysaccharides/hydrolyzed collagen layers on the silica particles surface was confirmed by EDAX analyses.



a b

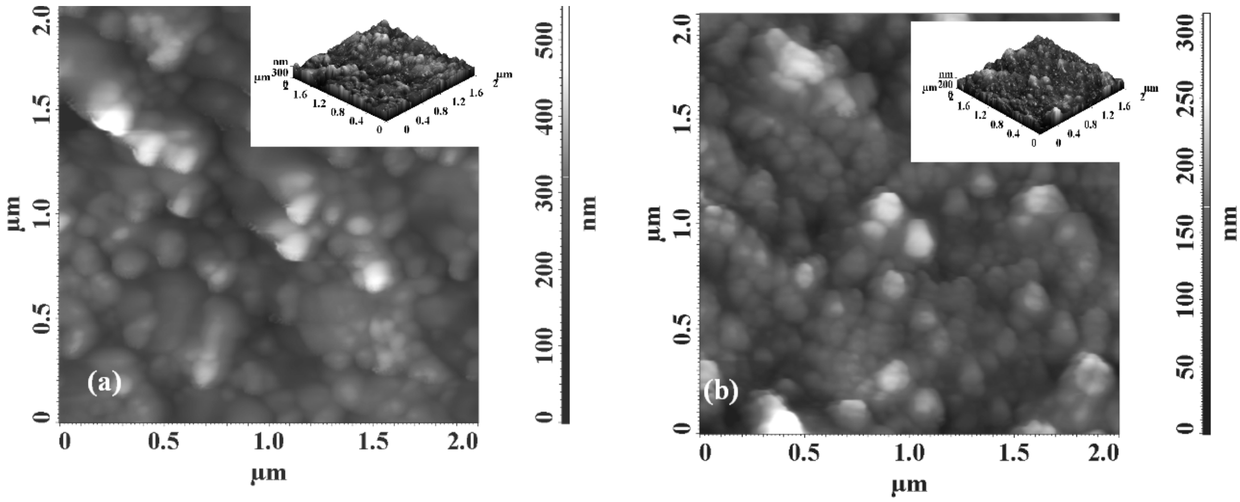
Fig. 4. Scanning electron micrographs (surface details) of silica microparticles (a) and silica with $(HC/PCurd)_4$ (b)

The elemental analysis of the silica microparticles with $(HC/PCurd)_4$ layers, investigated by EDX spectroscopy (Table 1) shows the presence of carbon (11.82 %) and phosphor (0.18%) atoms from the polymer layers deposited onto the MS surface.

Table 1. Elemental analysis of silica microparticles with $(HC/PCurd)_4$ layers.

Element	Wt, %	At, %
CK	7.25	11.82
OK	44.63	54.64
PK	0.29	0.18
SiK	47.83	33.36

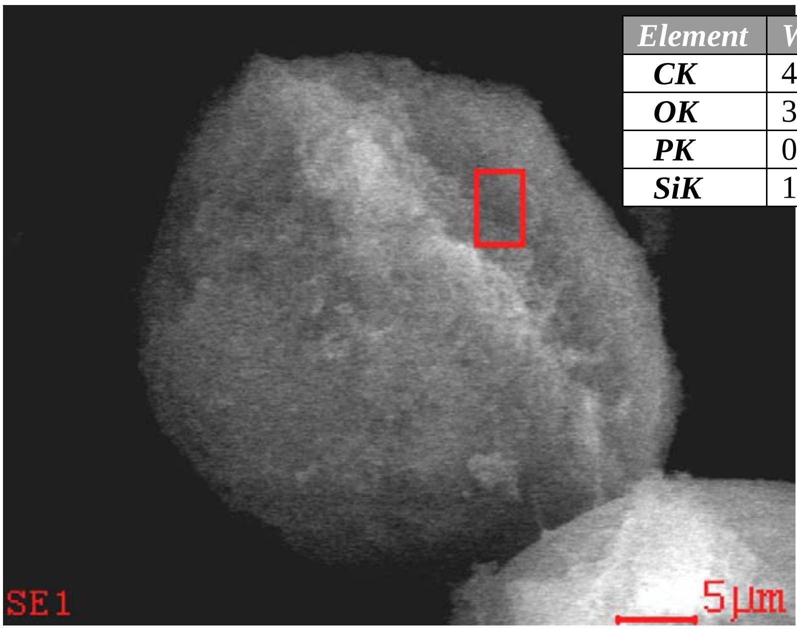
This change of the surface smoothness of the particles was confirmed by AFM (Fig. 5). Based on the topography images, a decrease of the root-mean-square surface roughness was observed from 62.18 nm for silica particles to 36.50 nm for silica with $(HC/PCurd)_4$.



R_a= 62.18 nm R_a= 36.50 nm

Fig. 5. AFM images of silica microparticle (a) and of a silica microparticle with $(HC/PCurd)_4$ (b), surface detail.

After the removal of the MS template by HF etching, the hollow capsules were obtained. The shell collapses during the dissolution of the silica core. This was evidenced by SEM-EDAX (Fig. 6). In the silica particles Si and O are the main elements due to the presence of SiO_2 (Table 1). After incubation in HF solution, the amount of Si decreases from 33.36 to 5.78 atomic percents and the carbon content increase from 11.8 to 55.95 atomic percents (Fig. 6) revealing that the core has been almost removed. The collapsing of microcapsules was also evidenced by particles size distribution presented in Fig. 7. The particles size slightly decreases from 49.44 µm to 44.6 µm after the removal of the silica core. The future exposure to HF did not reduce the silica content.



Element	*Wt%*	*At%*
CK	45.03	55.95
OK	36.94	34.47
PK	00.29	00.18
SiK	10.42	05.78

Fig. 6. Scanning electron micrographs of and EDX analysis of microcapsules with $(HC/PCurd)_4$

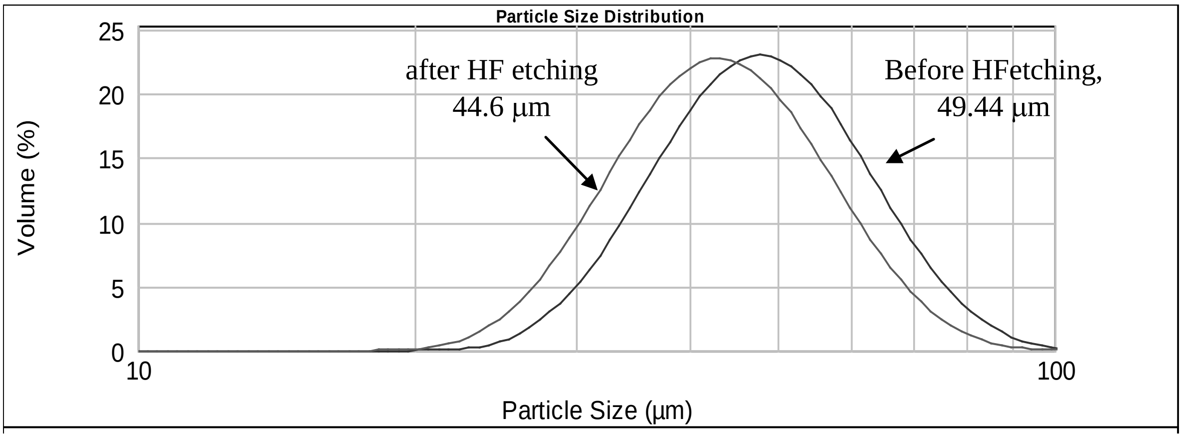


Fig. 7. Size distribution of silica microparticles with (HC/PCurd)4 before and after removal of the silica core.

Fig. 8a shows the thermo-oxidative decomposition of the silica particles covered with polyelectrolytes layers compared with the capsules. The removal of the silica core was confirmed by the increase of the total weight loss from 3% for SiO_2 covered particle to 8 % for the hollow capsules. Two weight loss processes were found in the TG curve of the capsules. The first weight loss (3%) occurred in the temperature range 80-170 °C and can attributed both to water evaporation and to decomposition of polysaccharide (PCurd)/polypeptide (HC) shell. The weight loss (6%) can be attributed to the oxidative decomposition of the polymers. The large residue amount can attributed to the residual SiO_2 from the core but also to the presence of the PCurd that also present a large residue amount at 700 C, as it can be seen in Fig. 8b.

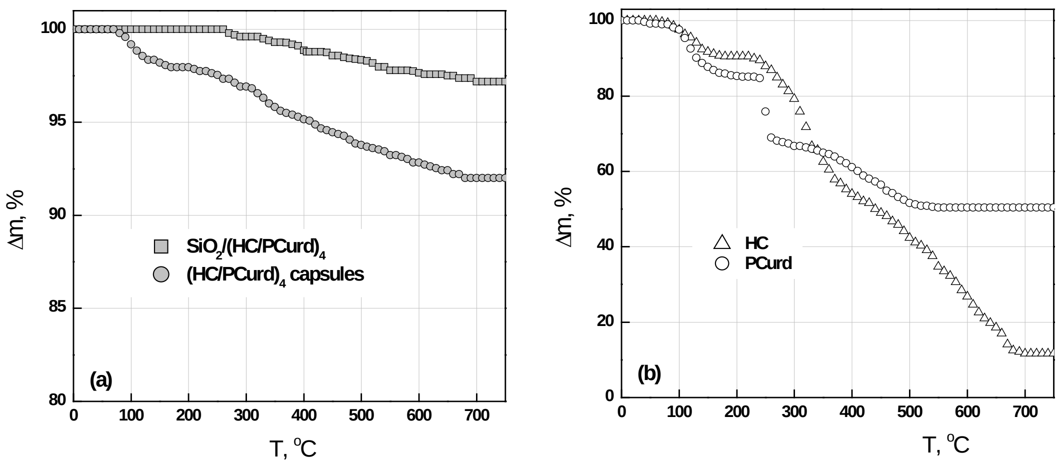


Fig. 8. TG curves for SiO_2/(HC/PCurd)$_4$ microspheres and (HC/PCurd)$_4$ capsules (a) and TG curves of the parent polymers (b)

4. Conclusions

The polysaccharide/polypeptide capsules were obtained by LbL assembly of four pairs of hydrolyzed collage and monobasic curdlan phosphate on silica particles. The LbL deposition was monitored by ζ-potential measurements and optical microscopy. ζ-Potential measurements of the multilayer coated particles indicated that the multilayer surface was being charged overcompensated in each adsorption step, facilitating the adsorption of the next oppositely charged polymer onto the MS spheres. The morphology of particles was investigated with SEM-EDAX and AFM techniques. The deposition of the multilayers on the silica particles was demonstrated by the decreased of the particle surface roughness observed from SEM images and measured by AFM microscopy.

The template was removed with 0.3 M HF at 4°C for 48h when the hollow capsules were obtained. The partial removal of the silica core was confirmed by EDAX analyses. The thermogravimetric analysis reveal an increase of the total weight loss from 3% for SiO_2 covered particle to 8 % for the hollow capsules that confirms once more the removal of the silica core. These hollow microcapsules obtained from LbL deposition of hydrolyzed collagen and a biodegradable polysaccharide can be used in future studies for encapsulation of active principle for medical and pharmaceutical applications.

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