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Protein loading into porous $CaCO_3$ microspheres: adsorption equilibrium and bioactivity retention

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Abstract

Formulation of proteins into particulate form is a main strategy to achieve controlled and target delivery as well as to protect fragile protein molecules. Control over size, mechanical properties, surface area (porosity) of particulate proteins has been successfully achieved by hard templating at mild conditions using porous $CaCO_3$ microspheres. A crucial step in this approach defining protein content is the loading of proteins into the $CaCO_3$ microspheres. In this study an adsorption of different proteins into the microspheres is investigated. Proteins with different characteristics such as molecular weight and charge were employed: catalase (Cat), insulin (Ins), aprotinin (Apr), and protamine (Pro). Thermodynamics of adsorption equilibrium is studied together with quantitative and qualitative analysis of protein loading and distribution in the microspheres. Protein interaction with the $CaCO_3$ microspheres is not limited by diffusion of protein molecules (protein dimensions are significantly smaller than microsphere pores) but is determined by protein affinity to the microsphere surface. Cat and Ins bind much stronger to the microspheres than Apr and Pro that can be explained by electrostatic attractive forces. Protein binding/release and protein biological activity are investigated as a function of pH. It is shown that pH variation during adsorption process plays a main role and defines not only an amount of protein adsorbed/released but also protein biological activity. Protein adsorption and microsphere elimination step (by EDTA) do not affect protein bioactivity. In addition to applications for protein particle/capsule formulations the findings of this study might help in understanding of protein interaction with carbonate minerals such as calcium carbonate used as natural material for multiple applications.

1. Introduction

The number of proteins and peptides used as therapeutic agents is significantly increasing due to a progress in modern biotechnology.[1] This stimulates to develop new approaches for nano- and microencapsulation of protein-based substances. Special attention is paid to protein encapsulation at mild conditions to keep protein activity. Such methods allow not only to perform encapsulation at mild conditions but also to control well particle shape and size. For this purpose the sequential adsorption of polymers or so-called layer-by-layer (LbL) deposition on decomposable solid matrices solutions is one of the most popular methods nowadays.[2-5] Protein immobilization into polymer nano- and microparticles can be performed by three different approaches as reviewed elsewere:[5] i)inclusion into solid decomposable matrices for further polymer LbL coating, ii) protein is one of LbL deposited polymers, and iii) loading of proteins into performed polymer particles. One can adjust a size and shape of the protein-containing particles by size and shape of used decomposable matrices or polymer particles.[5-9] For instance, salted out protein aggregates or insoluble protein-polyanion complexes may be used as matrices for further LbL deposition to form protein-containing particles with rather high polydispersity (particle size in the range 3-10 μm). These particles aim to be utilized for oral delivery where particle size distribution is not a critical issue.

Decomposable inorganic and organic matrices such as microparticles from melamin formaldehyde, polystyrene, PLGA, silica oxide, carbonates have been used to fabricate protein-containing micro- and nanoparticles with controlled size and shape (spherical).[3, 5, 10-13] Pulmonary delivery is envisaged in this case. Ten years ago calcium carbonate vaterite microspheres have been developed and nowadays became one of the most popular decomposable matrices.[14, 15] Together with easy preparation procedure and low costs these microspheres are biocompatible and can be eliminated at mild conditions such as slightly acidic pH or EDTA.[16, 17] Another important feature of the microspheres is that they are mesoporous. About a half of their internal volume belongs to pores (pore size in the range 20-60 nm) offering a space for encapsulation of macromolecules such as proteins, hormones, enzymes, etc.[14, 17] Protein encapsulation into the pores of the microspheres can be achieved by the following approaches: co-precipitation (entrapment during the microsphere synthesis), infiltration by solvent exchange, and physical sorption is reported.[5, 18, 19]

Despite of progressive increase of the number of scientific reports devoted to protein encapsulation by means of the porous $CaCO_3$ microspheres,[20-25] there is no full understanding of the mechanism of protein binding to the microspheres. Protein activity after microsphere decomposition is also not well studied. The aim of this study is to investigate thermodynamical parameters of protein adsorption on

carbonate microspheres in order to understand protein interactions with the microspheres. Model proteins with different molecular masses and charges are employed for this purpose: insulin (Ins), catalase (Cat), aprotinin (Apr), protamine (Pro). In additions, biological activity of the adsorbed proteins and those encapsulated after the microsphere dissolution by EDTA are studied.

2. Experimental section

2.1. Materials

$CaCl_2$ - 99%, "Biomedicals" ICN, Inc., USA; Na_2CO_3 - 99,8%, «Pharma», Russia; Pro from salmon and Cat from bovine liver - "Sigma", Germany; Apr from bovine lung - preparation "Ingiprol"(60 % active center) Belmedpreparaty, Belarus; trypsin from bovine pancreas (40 U/mg, 61% active center) - "Fluka", USA; Hydrogen Peroxide - "Sigma-Aldrich", USA; N-benzoyl-L-arginine ethyl ester – "Sigma", USA; EDTA – "Reakhim", Russia. Human recombinant Ins zinc salt was kindly provided by the Experimental Biotechnology Plant of Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, FITC, isomer I – "Sigma", Germany. All the chemicals were used without further purification. Milli-Q (Millipore, USA) water was used for all the experiments.

2.2. Preparation of $CaCO_3$ microspheres

The preparation of $CaCO_3$ microspheres by mixing of $CaCl_2$ and Na_2CO_3 solutions has been described in detail elsewhere.[15, 16] Briefly, 3 mL of 1 M Na_2CO_3 solutions were rapidly added to 9 mL H_2O, 3 mL of 1 M $CaCl_2$ and thoroughly agitated on a magnetic stirrer (650 rpm) for 45 s at 20 ^oC. It is important to properly chose salt concentration as well as stirring time and stirring speed because all these parameters influence the size of the $CaCO_3$ microparticles.[18] After that the agitation was stopped, and the reaction mixture was left without stirring for 15 min, during which time the formed amorphous primary precipitate of $CaCO_3$ transforms slowly into spherical microcores. Finally, the particles were separated by centrifugation at 1000 g, turned back into a suspension with 2 mL H_2O and dried at 70° ^oC for 2 h. All salt solutions were filtered before use by Corning Inc. (RC 0.20 μm) filter.

2.3. Protein adsorption

The batch adsorption of model proteins on the mesoporous $CaCO_3$ microspheres (0.005-0.080 mg mL^{-1}) was performed in 0.05 M glycine buffer, pH 8-10 subjected to constant stirring. The proteins used were Pro, Apr, Ins, and Cat. The initial concentration of proteins ranged from 0.1 mg mL^{-1} to 2 mg mL^{-1}. The suspensions of the microspheres in protein solutions were incubated 2-60 min under stirring at 23 ^oC, then centrifuged for 5 min at 10000 g and supernatants were analyzed. The

concentration of Pro was studied using Lowry method.[26] The concentrations of Apr, Ins, and Cat were studied through UV spectroscopy (Lambda 35, "Perkin-Elmer") at wavelength of 280 nm. In the experimental assays, adsorption measurements were conducted at least in triplicate. For protein release experiments, the $CaCO_3$ microspheres with adsorbed protein were washed twice with the same volume of 0.05 M glycine buffer to remove loosely attached protein molecules.

The amount of adsorbed proteins at saturation conditions (equilibrium) was calculated using the following equation:

$$q_e = \frac{(C_0 - C_e) * V}{m} \quad (1),$$

where q_o is the adsorption capacity (mg g^{-1}), C_0 and C_e are the initial and equilibrium protein concentrations (mg mL^{-1}); V is the volume (mL) of the protein solution; and m is the mass (mg) of $CaCO_3$.

The following Langmuir isotherm equations have been used to fit experimentally obtained adsorption isotherms:

$$\frac{1}{q_e} = \frac{1}{q_m} + \frac{K_a}{q_m}\left(\frac{1}{C_e}\right) \quad (2)$$

$$q_e = q_m + K_a \frac{q_e}{C_e} \quad (3)$$

$$\frac{C_e}{q_e} = \frac{1}{K_a q_m} + \frac{C_e}{q_m} \quad (4),$$

where q_m is the amount of protein molecules adsorbed to form a monolayer (mg g^{-1}), K_a is the Langmuir adsorption equilibrium constant (mg g^{-1}). q_m and K_a have been calculated from the plots i) $1/q_e = f(1/C_e)$, ii) $q_e = f(q_e/C_e)$, iii) $C_e/q_e = f(C_e)$, which correspond to the equations described above.

Efficiency of protein incorporation was calculated using the following equation:

$$\eta = \frac{C_0 - C_e}{C_0} \quad (5).$$

2.4. FITC-labeling of proteins

0.1 mg mL^{-1} FITC solution in 0.5 M carbonate buffer (pH 9.0) was drop-wise added to 2 mg mL^{-1} protein solution in 0.5 carbonate buffer under stirring until FITC:protein molar ratio of 1:5 was

achieved. The resulting solution was incubated for 4 h in a dark place and dialyzed twice against 50 mM TRIS buffer (dialysis bags with cut-off 8-10 kDa).

2.5. Optical microscopy

$CaCO_3$ microspheres were visualized on optical microscope «Carl Zeiss, JENA» (Germany).

2.6 Confocal laser scanning microscopy (CLSM)

CLSM analysis was done using *Zeiss* LSM *510 Meta* (Zeiss, Germany) equiped with oil-immersion objective with 63x magnification and numerical aperture of 1.4. Standard filter settings for excitation and emission of FITC were used for a laser sources with wavelength of 488 nm. For studying of penetration of proteins into $CaCO_3$ microspheres 0.2 mL of 1 mg mL^{-1} FITC-labeled protein solution was added to 0.5 mg of dry $CaCO_3$ microspheres for imaging after 30 min of incubation.

2.7. Scanning electron microscopy (SEM)

For SEM analysis $CaCO_3$ microsphere suspension in water was placed on a glass slide followed by drying during 1 hour at 90 ^{0}C. The sample was then sputtered with gold and SEM analysis was conducted using a Gemini Leo 1550VP instrument at operation voltage of 3 kV.

2.8 Protein biological activity

Specific activity of Cat was measured by monitoring a rate of hydrogen peroxide decomposition.[27] Specific activity of Apr was measured by inhibition of trypsin using N-benzoyl-L-arginine ethyl ester as a substrate.[28] To analyze the activity of Apr and Cat, $CaCO_3$ microspheres were washed twice in 0.05 M glycine buffer solution after protein adsorption, then dissolved in an equimolar amount of 0.2 M EDTA solution. Retention of activity of Cat and Apr has been calculated as a ratio between activities of the proteins after adsorption into $CaCO_3$ spheres followed by the sphere dissolution to activity of initially taken proteins.

2.9 Measurement of the hydrodynamic radius of proteins

Sizes of proteins were determined by photon correlation spectroscopy (DLS) using zeta-sizer (Nano ZS, Malvern, UK). 1 mL of filtrated 1 mg mL^{-1} protein solution in 0.05 M glycine buffer (pH 9.0) was injected into the cell and measured for 9 min.

2.10. Measurement of zeta-potential

The zeta-potential of proteins and of $CaCO_3$ microspheres was measured by dynamic laser light scattering method on zeta-sizer (Nano ZS, Malvern, UK). 1 mg mL^{-1} of protein or 0.5 mg mL^{-1} of $CaCO_3$ suspension in 0.0125 M glycine buffer (pH 9.0) was injected into a measuring cell and data were taken during 30 s.

2.11. Liquid Penetration Chromatography

Gel filtration of proteins was carried out using the Smartline system ("Knauer", Germany) using a high-pressure column packed with Biofox 17 SEC 9 (8 x 30 mm), previously calibrated with standard proteins: cytochrome C (M_w 12.4 kDa), carbonic anhydrase (M_w 29 kDa), bovine serum albumin (M_w 66 kDa) and amylase (200 kDa). The column was loaded with 0.5 mL of 0.5 mg mL^{-1} protein in 0.05 M glycine buffer (pH 9.0), the process was conducted at a flow rate of 0.5 mL min^{-1} in 0.05 M glycine buffer (pH 9.0). The absorbance was monitored at 280 nm.

3. Results and discussion

$CaCO_3$ microspheres with average diameter of 4.8 μm and low degree of aggregation were obtained by crystallization from supersaturated solution according to previous reports.[15, 16] As can be seen from Fig. 1, the microspheres have highly developed porous internal structure. The microspheres are composed from channel-like spherulitic nanocrystals of a size of tens of nanometers. As reported from previous study,[14] the average specific surface area of the microspheres is 8.8 m^2g^{-1}, their density is 1.6 g cm^{-3}, and an average pore size is in the range 20-60 nm. The particles used in this study have been prepared using the same protocol and have the same properties. Later these parameters will allow us to estimate the area occupied by adsorbed proteins. The pI of $CaCO_3$ microspheres is in the range 8-9.[16] Zeta-potential of $CaCO_3$ microspheres in 0.05M glycine buffer at pH 9.0 was found to be slightly positive (5.0±0.3 mV), that might be due to vicinity to the pI.

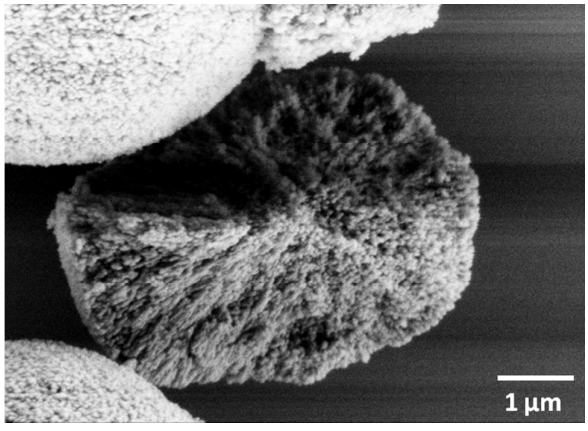

Fig. 1. SEM image of broken CaCO3 microsphere.

Further we focus on protein interaction with the porous carbonate particles to understand the mechanism of protein adsorption to the particles because both protein affinity to the particle surface and sterical limitations to diffuse through the pores may play significant role for the protein loading into the particles. Table 1 represents physical-chemical properties of proteins used in this study. Molecular weights, diameters known from literature and measured in glycine buffer as well as isoelectric points (pI) are included in the table. The glycine buffer has been incubated with $CaCO_3$ microspheres (40 mg/ml) followed by microsphere separation by centrifugation. This insures that conditions of measurements of protein solutions are equal to conditions of protein adsorption to the microspheres. As found by DLS measurements Pro, Apr, и Cat are present in solution as monomers and Ins as a hexamer. Gel-filtration chromatography confirms that Ins is mostly present as a hexamer (Fig. S1). One has to note that hydrodynamic diameter of the three proteins (Pro, Apr, and Ins) are similar to each other (3-4 nm) and that of Cat is much larger (15 nm) as shown in Table 1. Zeta-potential of the used proteins have been measured by DLS and the results are presented in Table 1. Pro is positively charged and Apr possesses rather low negative charge. In contrast, the zeta-potentials of Ins and Cat are much higher with negative sign.

Table 1. Physical-chemical properties of model proteins used in this study. Measurements of zeta-potential and hydrodynamic diameter are carried out in 0.05 M glycine buffer (pH 9.0) and in 0.05 M glycine buffer (pH 9.0) incubated with $CaCO_3$ microspheres (40 mg/ml), respectively.

Pro-tein	Mw, kDa	Hydrodynamic diameter, nm		pI	Zeta-potential,mV
		Literature data	Experimental data		
Pro	5.0	3.0[29]	4±1	11.0	2.0±0.3
Apr	6.5	2.9[30]	3±1	10.5	-4.2±0.3
Ins	5.8	2.7 (monomer), 5.1 (hexamer)[31]	4±1	5.3	-14.1±0.3
Cat	250	10.5[32]	15±1	5.4	-9.9±0.3

Firstly adsorption isotherms have been studied at room temperature. For this rather high concentration of $CaCO_3$ microspheres (40 mg/ml) and long incubation time (30 min) have been chosen because at this conditions one may load enough protein molecules and microspheres are saturated with a protein (Fig. S2, S3). The Langmuir isotherm is usually used to describe adsorption of a solute from a liquid solution as follows[33]:

$$q_e = \frac{q_m K_a C_e}{1+K_a C_e} \quad (6),$$

where q_e is the equilibrium adsorption capacity (mg g^{-1}), *C*e is equilibrium protein concentrations (mg mL^{-1}), q_m is maximum adsorption capacity or the amount of molecules adsorbed to form a monolayer (mg g^{-1}), K_a is the Langmuir adsorption equilibrium constant (mL mg^{-1}).

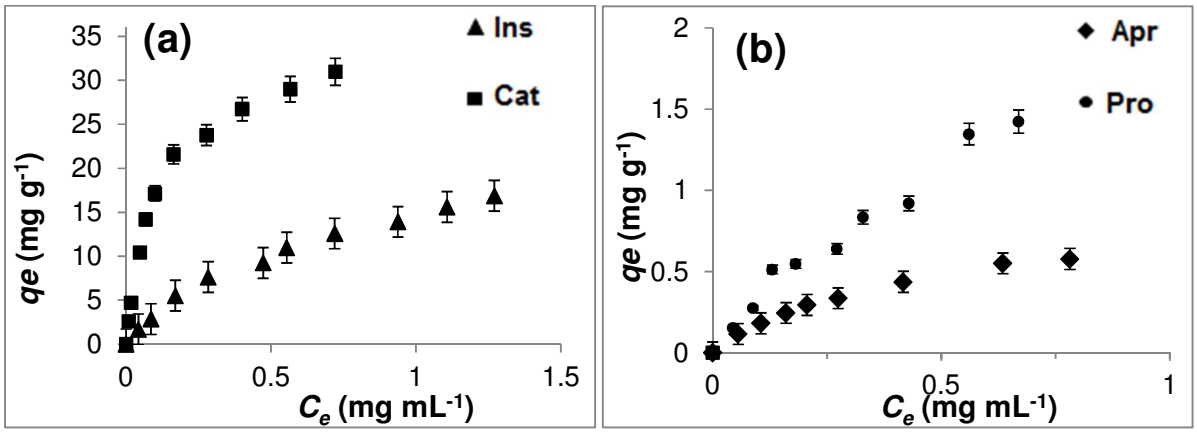

Fig. 2. Adsorption isotherms for Ins and Cat (a) as well as Pro and Apr (b) on $CaCO_3$ microspheres. In glycine buffer, pH 9.0. Concentration of $CaCO_3$ is 40 mg mL^{-1}, incubation time 30 min.

To obtain q_m and K_a, the experimentally found adsorption isotherms have been presented in three different ways. They correspond to the following functions: $1/q_e = f(1/C_e)$, $q_e = f(q_e/C_e)$, and $C_e/q_e = f(C_e)$.The resulted graphs on example of Cat fitted to linear curves are shown in Fig. 3a, 3b, and 3c, respectively.

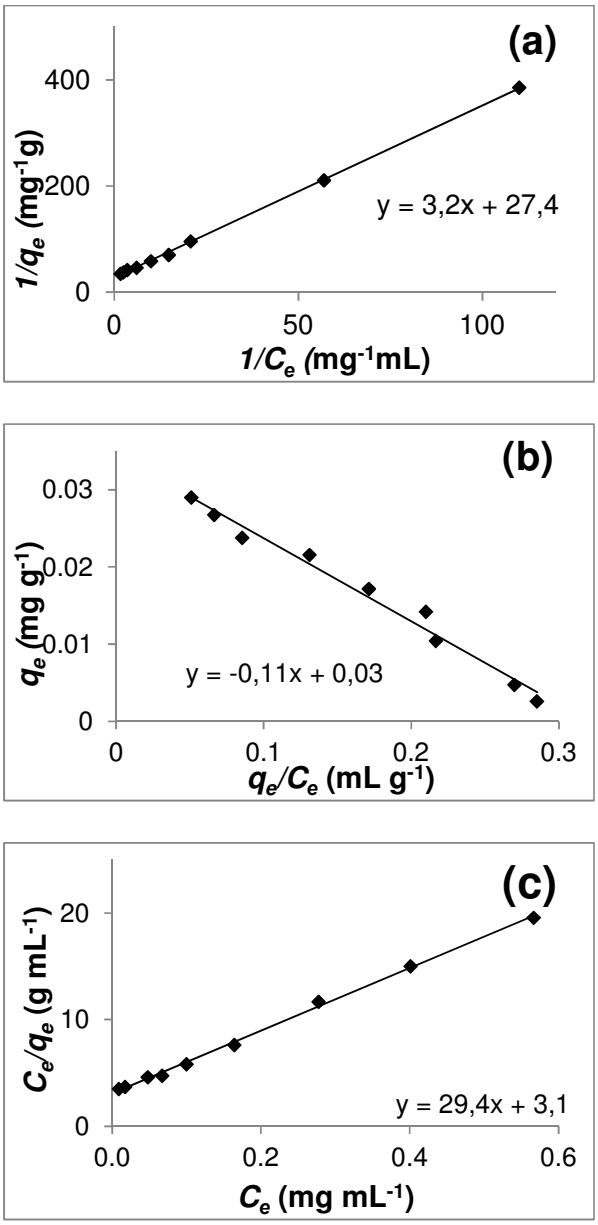

Fig. 3. Representation of data of adsorption isotherms of Cat as the following functions: $1/q_e = f(1/C_e)$ (a), $q_e = f(q_e/C_e)$ (b) и $C_e/q_e = f(C_e)$ (c).

The coefficient of determination was calculated for the three plots for all model proteins used in this study in order to find best correlation of experimental data:

$$r^2 = \frac{\sum(q_m - \overline{q_e})^2}{\sum(q_m - \overline{q_e})^2 + \sum(q_m - q_e)^2} \quad (7),$$

where q_m is the equilibrium capacity obtained from the isotherm model, q_e is the equilibrium capacity obtained from experiment, and $\overline{q_e}$ is the average of q_e.

Table S1 contains the calculated q_m and K_a and r^2 for the proteins used. To compare adsorption parameters for four model proteins, the best fitting (highest r^2) has been chosen for each and every protein. Table 2 shows q_m, K_a, and $1/K_a$ for the proteins.

Table 2. Parameters of protein adsorption (q_m, K_a, and $1/K_a$) found for best fitting to Langmuir equation (Table S1) as well as free Gibbs energy (ΔG) for model proteins used in this study.

Protein	q_m		K_a, L mol^{-1}	K_a^{-1}, mol L^{-1}	ΔG, kJ mol^{-1}
	mg g^{-1}	mol g^{-1}			
Pro	3.4±0.5	$(6.9\pm0.5)\cdot10^{-7}$	$(5\pm1)\cdot10^{3}$	$(1.9\pm0.2)\cdot10^{-4}$	-21±1
Apr	1.1±0.3	$(1.7\pm0.3)\cdot10^{-7}$	$(11\pm2)\cdot10^{3}$	$(0.9\pm0.1)\cdot10^{-4}$	-23±1
Ins	23.0±2.0	$(6.5\pm0.5)\cdot10^{-7}$	$(60\pm5)\cdot10^{3}$	$(1.7\pm0.2)\cdot10^{-5}$	-27±1
Cat	34.0±2.0	$(1.5\pm0.2)\cdot10^{-7}$	$(2380\pm150)\cdot10^{3}$	$(4.2\pm0.5)\cdot10^{-7}$	-36±2

Maximum adsorption capacity (q_m) in protein mass is much higher (more than order of magnitude) for Cat and Ins if compared to Pro and Apr (Table 2). However, if considering this value as a number of protein molecules adsorbed, there is no significant difference between these four proteins (Table 2). This means that a number of adsorption centers for all the model proteins studied is similar. Larger proteins such as Cat and Ins (hexamer) show higher adsorbed amount in terms of mass.

Adsorption equilibrium constant K_a was also much higher for Cat and Ins compared to other proteins. As a result, free Gibbs energy ΔG calculated by the equation below has been lower for Cat and Ins indicating higher affinity of these proteins to microsphere surface compared to Apr and Pro:

$$\Delta G = - RTlnK_a \qquad (8),$$

where R is universal gas constant (8.314 J mol^{-1}K^{-1}) and T is the absolute temperature in K.

Negative values of ΔG means that for all the four model proteins equilibrium in protein adsorption is shifted towards adsorbed protein molecules. $1/K_a$ represents an equilibrium concentration of protein molecules when a half of adsorbed centers are occupied. These values are much lower for Cat and Ins compared to Apr and Pro. Thus, affinity of Cat and Ins to carbonate microspheres are

higher than that of Apr and Pro. We believe that the higher affinity of Cat and Ins that Pro and Apr can be explained by an impact of electrostatic forces involved into the adsorption process. Cat and Ins are negatively charged that may drive their interaction with positively charged $CaCO_3$ microspheres. In contrast, Apr has very low but negative zeta-potential and Pro has positive zeta-potential that may reduce the interaction of these proteins with the microspheres by electrostatic binding.

Porous structure of the $CaCO_3$ microspheres allows one to load large amount of adsorbing molecules due to high surface area available for mesoporous structure. We have evaluated an input of the porous structure to the amount of adsorbed proteins. Fig. 4 presents theoretical values of maximum protein adsorption assuming monolayer formation for non-porous (gray) microspheres and porous (light gray) ones used in this study (diameter of 4.8 μm and surface area of 8.8 m^2/g).[16] Surface area occupied by a protein molecule has been chosen to be a circle with diameter equal to hydrodynamic diameter determined in this study by DLS (Table 1). The capacity for maximal theoretical protein adsorption for porous microspheres is much higher (about an order of magnitude) than to non-porous due to large surface area. Experimentally found amount of proteins adsorbed in porous microspheres (Fig. 4, black columns) was found always below theoretically calculated maximal values. However, the experimentally found values differ from one protein to another. Ins and Cat occupied 94 and 82% from theoretically maximal values and Apr and Pro just 8 and 33%, respectively. To understand why Apr and Pro have much lower affinity to the carbonate microspheres we have further studied protein penetration into microspheres and their distribution inside the microspheres.

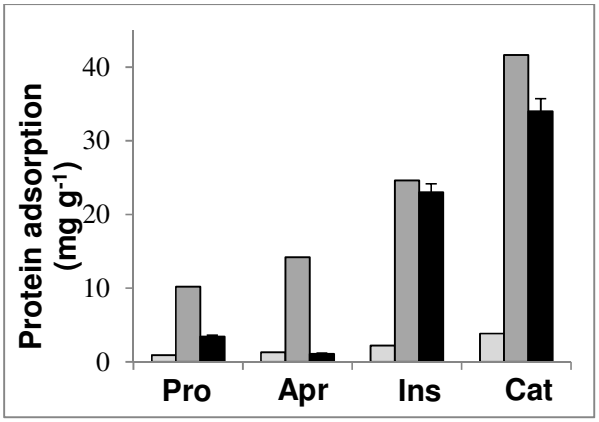

Fig. 4. Maximum protein adsorption calculated for porous (light gray) microspheres used in this study and non-porous (gray) microspheres with the same diameter (4.8 μm). The measured amount of proteins adsorbed (q_m) on porous microspheres is presented as black columns.

To follow protein diffusion into the microspheres and protein distribution inside the microspheres we have used CLSM. For this analysis all the four proteins have been labeled by FITC by the procedure described elsewhere.[34] However, Apr-FITC and Pro-FITC have been aggregated in the presence of supernatant of $CaCO_3$ probably because of low colloidal stability of the proteins in the pH range close to their pI (pH 9, glycine buffer). Another explanation can be the following. The aggregation may be caused by significant changes of the hydrophilic-hydrophobic balance of these rather small proteins even in case if one protein molecule gets one molecule of FITC during the labeling procedure. Cat and Ins (hexamer) are much larger and one FITC molecule does not change the balance significantly. Ins and Cat have been stable and their interaction with $CaCO_3$ microspheres has been studied. Both proteins are distributed inside the whole volume of the $CaCO_3$ microspheres indicating no diffusion limitations to diffuse through the microsphere pores (Fig. 5). However, Ins has been distributed more homogeneously inside the microspheres compared to Cat. This may be explained by larger size of Cat molecules (hydrodynamic diameter of 15 nm against 4 nm of Ins). Thus, some number of pores is not available for Cat molecules (pores in the range 20-60 nm), however larger pores do not present any diffusion barrier and Cat molecules can penetrate through the whole volume of the microspheres. We believe that no diffusion limitation should take place for unlabelled Apr and Pro because their size is similar to the size of Ins molecules. Very low occupation of microsphere molecules by Apr and Pro compared to Ins and Cat may be explained by low affinity of Apr and Pro to carbonate surface but not by diffusion limitations for penetration through the microsphere pores. This fact is supported by lower K_a and higher free Gibbs energy for Apr and Pro compared to Ins and Cat.

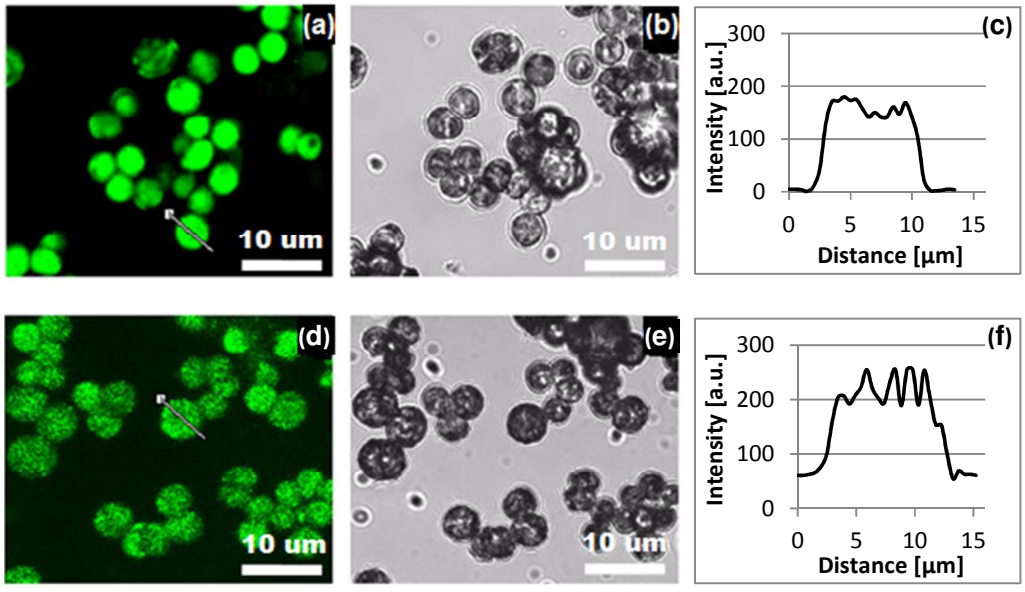

Fig. 5. C LSM images of $CaCO_3$ microspheres after incubation with Ins-FITC (a, b, c) and Cat-FITC (d, e, f). (a, d) – fluorescent images, (b, e) – transmission images, (c) and (f) - fluorescence profiles for the image (a) and (d), respectively.

Binding of the adsorbed proteins can be studied by considering an amount of the adsorbed protein molecules washed out after rinsing with buffer solution. Table 6 shows the amount of protein included in the microspheres after adsorption and the amount after two washing steps in buffer solution. Protein retention has been calculated from these values. Cat has demonstrated very strong binding to the microsphere surface and was almost not released after washing. Other proteins have been released more significantly probably because of lower (weaker) binding compared to Cat. Pro and Ins have also been retained to high extent in the microspheres after washing that may be caused by large size of Ins hexamer and rod-like structure of peptide Pro. Free Gibbs energy of Apr, Ins, and Pro are about two times higher than that of Cat. This may explain strong binding of Cat which has almost not been washed out after washing procedures.

Retention of protein biological activity after elimination of decomposable $CaCO_3$ microspheres is crucial for protein encapsulation using the microspheres. Cat kept about 80% of its initial activity after adsorption into the microspheres followed by the microspheres dissolution in 0.2M EDTA (Table 6). Taking into account that Cat does not change its activity in solution in the presence of 0.2M EDTA (data not shown), the reduction of Cat activity by 20% might be related to partial protein denature either due to adsorption onto the carbonate surface or rather high pH at adsorption conditions (pH 9.0). We have tested the effect of pH on Cat activity in solution and found the activity of Cat to be pH dependant (Fig. 6). After 1h incubation Cat activity has been reduced almost twice in pH 9.0. This means that a change of Cat secondary structure is not significant after adsorption onto $CaCO_3$ microspheres and further microsphere dissolution, the protein looses activity due to rather high pH values during the adsorption experiment. Apr has kept fully its biological inhibition activity after adsorption followed by microsphere dissolution in 0.2M EDTA. Apr activity does not depend on pH (Fig. 6). This again proves that adsorption of these proteins (Cat and Apr) followed be their release (microsphere decomposition) does not significantly affect the protein secondary structure. This is very important and shows that decomposition by EDTA might be used to eliminate carbonate microspheres aiming at protein encapsulation by $CaCO_3$ microsphere templating.

Table 6. Characteristics of protein adsorption in CaCO3 microspheres and activity retention after the microsphere dissolution.

Protein	Protein content, mg g^{-1}		Protein retention after two washing steps, % from adsorbed protein	Retention of specific activity after dissolution of $CaCO_3$ in EDTA, % of initial
	After adsorption*	After two washing steps		
Pro	1.5±0.1	1.1±0.1	74	-
Apr	0.6±0,1	0.10±0.02	16	100 ± 4
Ins	11.0±1.0	7.0±1.0	64	-
Cat	20.3±1.0	20.0±1.2	99	79 ± 5

*Adsorption conditions: $CaCO_3$ and protein concentration are 40 and 1 mg mL^{-1}, respectively, incubation time 30 min, glycine buffer pH 9.0.

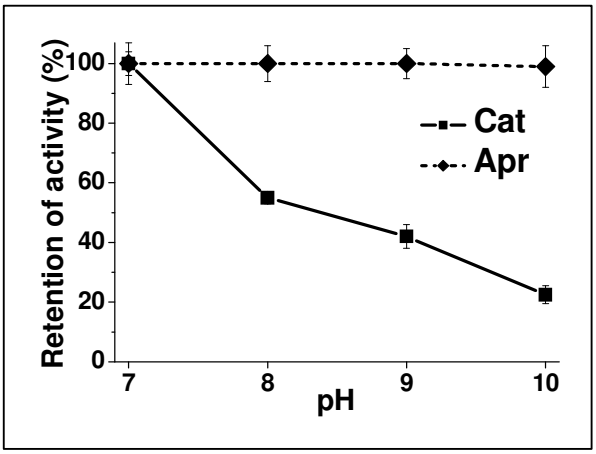

Fig. 6. Retention of specific activity Cat and Apr after 1 h incubation in glycine buffer with pH adjusted in the range from 7 to 10. Protein concentration 1 mg mL^{-1}.

pH has strong effect not only on protein activity but also on protein adsorption. Further we have studied how incubation pH affects protein binding and subsequent protein release during washing step. This is important for encapsulation procedure because washing out of poorly bound protein molecules or layer-by-layer polymer coating of protein-containing $CaCO_3$ microspheres includes multiple washing steps. For higher pH values (range from 8 to 10) the amount of proteins adsorbed has been decreased for all proteins except for Cat (Fig. 7a). Probably very strong binding of Cat compared to other proteins (indicated by much lower free Gibbs energy, Table 2) makes Cat adsorption pH independent. The amount of protein remained after adsorption followed by washing with buffer is presented in Fig. 7b. For the most of proteins an amount of protein released after washing increased for

higher pH values. This means that there is direct correlation between protein binding and release as a function of pH. The stronger is the protein binding, the less protein molecules will be released after washing step. If a protein is bound very strongly such as Cat in this study, its binding and release might be pH independent. In general, one can consider the $CaCO_3$ particles as matrices to separate proteins by employing different affinity of the adsorbing protein to the particles at various pH (in the range above neutral pH to keep the particles stable). This may give an option for reuse the particles and make use of their developed surface area for adsorption of high amount of proteins.

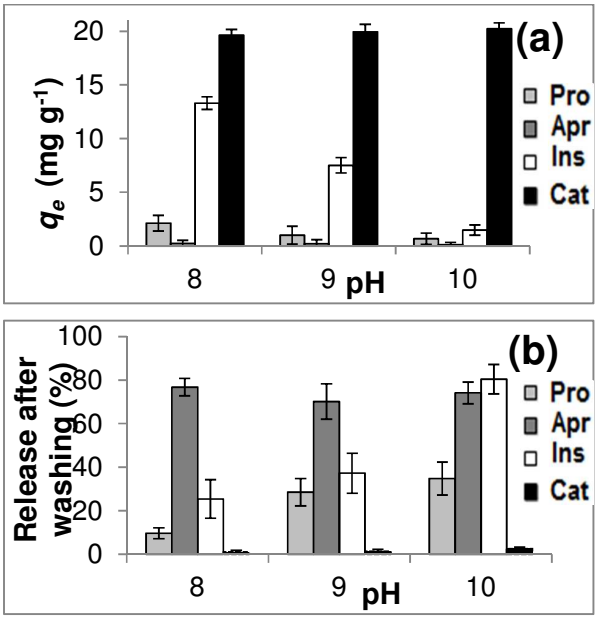

Fig. 7. The amount of protein adsorbed in $CaCO_3$ microspheres (a) and released after subsequent washing procedure (b) as a function of pH.

Conclusions

Protein adsorption on porous vaterite $CaCO_3$ microspheres has been investigated for the following proteins having different molecular weights and isoelectric points: Pro, Apr, Ins, and Cat. Adsorption isotherms have been constructed and parameters of adsorption equilibrium such as q_m, K_a, ΔG have been calculated. The adsorption isotherms fit well to the model of monomolecular Langmuir adsorption. Negative values of free Gibbs energy were found to be in the range from -40 to -20 kJ mol-^{1}. This shows that the adsorption equilibrium for all the studied proteins is shifted towards the adsorbed protein. Cat and Ins have lower values of ΔG that may explain why these two proteins have stronger interaction with the microspheres. Ins and Cat occupied 94 and 82% from theoretically calculated monolayer and Apr

and Pro just 8 and 33%, respectively. This means that protein adsorption is not limited by sterical hindrance of protein diffusion through mesoporous structure of the microspheres but depends on protein affinity to the carbonate surface. No diffusion limitations may take place because the dimensions of pores of the microspheres (20-60 nm) are larger than hydrodynamic diameters of the proteins (15 nm for Cat and about 4 nm for other proteins studied). Cat has the highest affinity to the microspheres (lowest ΔG of -36 kJ/mol) and almost was not released after adsorption followed by multiple washing steps; other proteins show release of 30-70% of initially adsorbed amount. Moreover, the stronger is the protein binding, the less protein molecules will be released after washing step as found in the pH range 7-10. Proteins (Cat and Apr) keep their biological activity after adsorption followed by the microsphere dissolution in EDTA. This demonstrates high promises of $CaCO_3$ microspheres as templates for protein encapsulation.

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