1. Introduction

Polyphenol compounds originated from plants possess a high spectrum of biological activity including antioxidant, antibacterial, antiviral ones, and others [1–3]. Utilization of nanoscale vehicles loaded with polyphenols instead of free compounds can improve bioavailability and half-life of the compound in vivo and in vitro, optimize routes of their administration, and achieve site specific delivery combined with controlled release properties to be constructed with nanometer precision and predetermined layer composition via sequentially saturating adsorption of a layer of each component on a template surface [20–27]. Among the species used for LbL assembly are polyelectrolytes, nanoparticles, DNA, and enzymes as well as several low molecular weight substances, such as porphyrins [22]. LbL-constructed multilayers on the basis of tannic acid (TA), or pentagalloyl glucose, with typical artificial polyelectrolytes poly(allylamine hydrochloride), polydiallyldimethylammonium chloride [20], 90% quaternized poly(N-vinylpyrrolidone) [27], naturally derived polyelectrolyte chitosan [21], as well as several uncharged polymers, such as poly(N-vinylcaprolactam), poly(N-vinylpyrrolidone), poly(ethylene oxide), poly(N-isopropyl acrylamide) [27], have been investigated as potential materials with pH-controllable solubility for sustain release formulations. The kinetics of adsorption of (−)-epigallocatechin gallate (EGCG), tannic acid, tearubigins (pigments of black tea) on an immobilized layer of bovine serum albumin have been studied using quartz crystal microbalance techniques [28–30].

According to authors’ knowledge, layer-by-layer assembly of a polyphenol and a proline-rich protein has not been investigated. We choose (−)-epigallocatechin gallate (Fig. 1) as one of the components of the assembly because of its well-known health benefits,
2. Materials and methods

2.1. Materials

(−)-Epigallocatechin gallate from green tea (EGCG), gelatin, type A from porcine skin (GelA, ∼300 Bloom), gelatin, type B from bovine skin (GelB, 225 Bloom), sodium chloride, hydrochloric acid, sodium hydroxide, potassium persulfate (K2S2O8), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), tetramethylrodamine-5-isothiocyanate (TRITC) and fluorescein isothiocyanate (FITC) were used purchased from Sigma-Aldrich and used as received.

Fluorescently labeled gelatins, GelA-FITC and GelB-TRITC, were prepared as in Refs. [34,35] and the labeled proteins were separated using a Amersham Bioscience PD-10 desalting columns.

2.2. Film deposition

(GelA/EGCG)n and (GelB/EGCG)n (n = 0.5–10) films were assembled by sequential dipping of 0.9 × 1.8 × 0.09 cm² quartz slides into 3.0 mg/mL aqueous solutions of a gelatin (GelA or GelB) and EGCG at pH 6.5 for 15 min per layer with three intermediate washings in deionized (DI) water.

The thickness of the deposited gelatin/EGCG multilayers was estimated by the Quartz Crystal Microbalance (QCM) technique. The frequency changes of resonators were monitored using a USI-System, Japan QCM instrument with the accuracy of ±1 Hz and converted into thickness using experimental scaling with the Sauерbrey equation: ΔD(nm) = −0.016ΔF(Hz). The mass of deposited material was recalculated from the frequency fall using the following equation: Δm(ng) = −0.84ΔF(Hz). The detailed description of the procedure can be found elsewhere [20,21]. A precursor layer of gelatin was preliminary adsorbed on all resonators. After adsorption of each layer, the resonators were washed in an excess amount of DI water and dried in the stream of nitrogen. For gelatin A, two separate sets with two resonators in each were done. In order to find out if the longevity of solution usage can affect the deposition step, the adsorption on one of the resonators that, the particles were separated from the supernatant and used previously to produce polyelectrolyte capsules [21]. MnCO3 microcores with a diameter of 4.0 μm (∼0.2 g) were dispersed in 2 mL DI water using a bath sonicator, water was changed twice using centrifugation at 3500 rpm to precipitate the particles. No precursor polyelectrolyte layer was formed on microparticles. The first layer was always that of gelatin. The total volume of microparticles suspension was readjusted to 1.0 mL and 0.5 mL of a 3 mg/mL gelatin solution (pH 6.5) was added for 15 min. After that, the particles were separated from the supernatant and washed three times with DI water. A EGCG layer was formed by adding 0.5 mL of a 3 mg/mL EGCG solution per 1.0 mL of the suspension of gelatin-coated microparticles. Fifteen minutes later, the microparticles were separated by centrifugation and rinsed with DI water. The assembly was monitored with surface potential (ξ-potential) measurements using a Brookhaven Zeta Plus microelectrophoretic instrument. For the measurements, 20 μL of sample solution were re-dispersed in 2 mL DI water and the readings were taken.

To prepare four bilayer GelA/EGCG capsules, 0.25 mL of microcores with GelA/EGCG shells was mixed with 0.75 mL of a 0.05 M EDTA (pH 6.5) for 3 days, and than pH was adjusted to 1.0 for 30 min. After visual core dissolution, the supernatant was removed and replaced with 1 mL of fresh 0.05 M EDTA. pH of the suspen-
sion was adjusted to 9.0 for 1 min, after that, the \((\text{GelA}/\text{EGCG})_4\) capsules were separated by centrifugation and washed three times with DI water to remove soluble salts. Utilization of a 0.05 M EDTA solution alone without the pH adjustment or a 0.1 M HCl solution without EDTA to dissolve the carbonate core did not allow to produce the capsules without core traces. None of the dissolution methods used gives stable \((\text{GelB}/\text{EGCG})_4\) capsules.

Atomic force microscopy (AFM) images of dried capsules on mica and films on silicon supports were taken using a Q-ScopeTM 250 Quesant instrument in an intermittent-contact mode. Confocal laser scanning microscopy (Leica DMI RE2) was used to analyze the structure of Gel/EGCG shells on cores and microcapsules after core dissolution. SEM images were taken on a Hitachi S-4800 scanning electron microscope equipped with an energy dispersive X-ray analysis (EDAX) accessory. To enhance image quality, sample surface was spattered with 2 nm layer of iridium.

2.4. Capsule permeability test

Fluorescein isothiocyanate and FITC-labeled dextrans (1 mg/mL, Sigma) with the molecular weights of 4400, 77,000, and 2,000,000 were used for permeability test experiments. Typically, 20 μL of a capsule solution with pH 6.5 was admixed to 20 μL of a dextran solution on a glass slide placed in the holder of the confocal microscope. One of the capsules was chosen, zoomed on, and the intensities of the light emitted by capsule interior \((I_{int})\) and by surrounding solution \((I_{ext})\) were measured 10 min after the mixing. The measurements were done for 20–25 unbroken capsules and averaged.

2.5. ABTS** assay

Stock solution of cation-radicals of ABTS (ABTS**+) was prepared as in Ref. [36]. 88 μL of a 0.14 M K3S2O8 was added to 5 mL of a \(7 \times 10^{-3}\) M ABTS aqueous solution and left overnight at room temperature. The obtained stock solution of intensive blue-green color was kept protected from light at \(+2^\circ\)C. The stock solution was diluted with DI water (pH 6.5) immediately before use in such a way that the absorbance at 734 nm \((A)\) was equal to 1.35 ± 0.05 \((l = 1.0 \text{ cm}, \epsilon = 1.4 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1} [36])\). The difference of \(A_0\) in various series was not higher than ±0.05.

To evaluate antioxidant properties of free EGCG, GelA, or GelB, 10–250 μL of 0.3–3.0 mg/mL solution of the components were added to 2.0 mL of the aqueous ABTS** solution (pH 6.5) as above under constant stirring. Absorbance of the mixture at 734 nm \((A_t)\) was followed in time for 60 min with the reading intervals of 10 s using an Agilent 8453 spectrometer. The influence of gelatin on EGCG determination with the ABTS**+ assay was evaluated in an additional set of experiments (Supporting information) and found to be moderate.

Quartz slides with \((\text{GelA}/\text{EGCG})_n\) or \((\text{GelB}/\text{EGCG})_n\) layers on them were placed into 2.0 mL of the ABTS**+ solution under stirring and changes of \(A_t\) were followed in time. The moment of slide immersion into solution was taken as \(t_0\). To evaluate the background change due to quartz slide immersion, the empty wet supports were placed into the ABTS**+ solution under experimental conditions and the \(A_{734}\) drop, typically 0.14 ± 0.05 a.u., was taken into account in further calculations.

3. Results and discussion

3.1. Layer-by-layer assembly on QCM resonators

The gelatin/EGCG multilayers show a stable tendency to grow with deposition of each layer (Fig. 2). Being equal to 5.0–7.0 nm for the first two bilayers, the thickness of GelA/EGCG bilayer increases up to 10 nm per layer when the number of deposition cycles reaches four and then uncontrollably, with a step higher than 30 nm (Fig. A, Supporting information). Almost similar results were obtained for all runs. Since the adsorption of GelA/EGCG layers on one of the resonators was started when 5 bilayers had been already adsorbed on another from the same solution, solution contamination or deterioration in the assembly process can be excluded as a reason of bulk film formation.

The total thickness of the GelB/EGCG multilayers is smaller than that of EGCG/GelA films with equal number of bilayers. At the same time, the GelB/EGCG bilayer thickness also has tendency to increase, although the step is not so prominent as compared with GelA/EGCG films. Several first GelB/EGCG bilayers have a 2–4 nm thickness, which increases to 6–8 nm for further layers (Fig. A, Supporting information).

According to numerous studies, polyphenol/protein binding is based mainly on hydrogen bonding between hydrophobic amino-
acid residues of proteins and phenol ring of polyphenols [1,13–19], but it is also affected by protein charge [28,29]. We assume that hydrophobic forces play the main role in the formation of gelatin/EGCG layers, since EGCG, unlike tannic acid [20], does not form stable Lbl assemblies with polycations. The fact that thickness of GelA/EGCG bilayers is higher than that of EGCG/GelB bilayers can be related with various physical chemical properties of the gelatin used (varying charge density and Bloom numbers). Most probably, the exponential growth of the layers is caused by adsorption of more than one layer of EGCG or gelatin per one deposition cycle. A partial release of EGCG from previously adsorbed layers into protein solution and an extra adsorption of EGCG into already deposited gelatin layers from its solution can favor formation of the bulk films. Protein binding to hydrophobic proteins is a complex reversible multistage process which is incompatible with a single structure. There are numerous binding sites even for a simplest polyphenol/protein system. The interaction starts from compacting protein molecules by polyphenols on the initial stages, proceeds trough formation of a dimer of polyphenol coated protein and finally leads to large complexes and protein precipitates [1,13–19].

3.2. Interaction of ABTS** with EGCG in solution

EGCG in solution shows a mixed scavenging activity toward ABTS cation-radicals. A short fast period completed within less than 20 s after adding EGCG to a ABTS** solution is followed by a long slow scavenging stage (Fig. B, Supporting information). Biphasic kinetics have been observed for the ABTS** reaction with several other antioxidants, among them complex flavonoid compounds, and attributed to scavenging by polyphenol oxidation products formed on early stages of the reaction [37–41]. For EGCG, the estimated radical scavenging activity (RSA), which shows how many ABTS** radicals react with one inhibitor molecule, reaches 11.5 ± 1.9 after 20 min. Taking into account, that for several polyphenols (including Trolox and gallic acid), around 1.92 ABTS cation-radicals can react with one –OH group [38] and one molecule of EGCG has eight such groups, one can calculate that after 20 min the reaction is completed to about 75%. After 24 h, the RSA value reaches 13.8 ± 0.7 (Fig. C, Supporting information). According to our results (Fig. D, Supporting information), the presence of 0.03–1.9 mg of gelatin per 1 mg of EGCG has only small influence on EGCG determination in the solution with the ABTS** assay after 20 min of reaction. The ratio of a EGCG concentration determined in the presence of gelatin to that in the solution without the protein is in range from 0.93 to 1.07 and such influence was neglected. Both gelatin A and gelatin B were found to contain less than 0.2 mmol –OH groups per 1 g; that is more than two hundred times less than EGCG does. The amount of ABTS** reacted with gelatin in the EGCG/gelatin mixtures and films was ignored in further calculation.

3.3. Interaction of ABTS** with gelatin/EGCG multilayers

For gelatin/EGCG films, the reaction of EGCG with the ABTS cation-radicals retains biphasic characteristics (Fig. 3a). However, the initial ABTS** scavenging rate (W) for films (Fig. 3) is several times lower than that in a solution (Wsolv ∼ 10−5–10−4 M/s). We assume that such phenomenon is related to inhibition of the reaction of ABTS** with EGCG by gelatin forming strong complexes with the polyphenol on initial stages [1]. For gelatin/EGCG films, the reaction of EGCG with ABTS** was not completed even after 60 min, a further slow decrease of cation-radical absorbance was always observed.

The averaged amounts of EGCG adsorbed in the EGCG/gelatin films were evaluated from absorbance change of a ABTS** solution 20 min after immersion of the corresponding film, assuming that RSA in the films is equal to that in solution. The EGCG content is about 272 and 89 ng/cm² per layer for multilayers of gelatin A and gelatin B respectively. The higher amount of EGCG in GelA-based layers correlates with the formation of thick GelA/EGCG bilayers as found by QCM.

To evaluate the percentage of EGCG in the multilayer material we estimated mass of a film with known amount of bilayers from QCM data and recalculated EGCG content in it. For example, the averaged mass of 7 GelA/EGCG bilayers from QCM data is 6.3 μg/cm² and averaged EGCG content is ca. 1.9 μg/cm². Therefore the film contains about 30% w/w of EGCG. For GelB/EGCG layers, the estimated value is higher than 28% w/w.

For gelatin/EGCG films, unlike that for tannic acid/polyallylamine multilayers [32,33], the value of W increases with increasing number of bilayers in the film and no plateau is observed on the graph (Fig. 3b). A possible reason for it can be an exponential growth of gelatin/EGCG layers as proven by QCM along with an extreme imperfection of the film structure.

According to AFM data (Fig. 4), the surface of a dried (GelA/EGCG)ₙₙ film is very rough, with numerous aggregates of a 300–500 nm diameter. At higher resolution (Fig. 4b), smaller complexes of gelatin A and EGCG with a size less than 100 nm are observed in the structure of the bigger aggregates. Similar but less rough
patterns were found for GelB/EGCG multilayers. They are characteristic for films both with EGCG and gelatin outermost layers. The hydrophobic complexes are formed in the course of LbL assembly of gelatin and EGCG but their size and shape could be affected by film collapse during drying. We assume that the parent wet gelatin/EGCG multilayers have a porous-like structure with plenty of holes and isolated islands of hydrophobic material and the diffusion of the ABTS$^{+}\cdot$ reagent in the film is not limited.

### 3.4. Gelatin/EGCG assembly on cores

Since both GelA/EGCG and GelB/EGCG bilayers can be formed on flat quartz supports, we tried to form shells consisting of gelatins and EGCG over MnCO$_3$ microparticles. A layer of corresponding gelatin was always adsorbed as the first one. The observed positive $\xi$-potential values for GelA and negative ones for GelB coated microparticles are in good agreement with isoelectric points of the gelatins (Fig. 5), while for the EGCG/GelB assembly, only light variation of a negative value was observed under experimental conditions (pH 6.5). A negative surface charge of the nanoparticles bearing an EGCG outermost layer seems to be in good agreement with the direct measurements of electrophoretic potential of EGCG in solution, according to which the polyphenol has an apparent $pK_a$ value between 3 and 4 [42]. The negative surface charge of EGCG-coated nanoparticles also correlates with data obtained by us previously for tannic acid [20], a compound which has similar structural elements.

Confocal images of gelatin/EGCG shells coated around MnCO$_3$ cores are presented in Fig. 6. To enhance the contrast of fluorescence images, GelA was preliminary labeled with FITC, and GelB with TRITC. (GelA-FITC/EGCG)$_4$ shells appear to be rather uniform, forming rings with equally distributed fluorescence, and therefore, with equally distributed components around the cores. At the same time, a non-uniform distribution of TRITC-labeled gelatin B for (GelB-TRITC/EGCG)$_4$ shells indicates the formation of a net of hydrophobic GelB/EGCG complexes around the cores, which, however, has a lot of defects. To exclude the possibility of bad attachment of gelatin B to negatively charged core surface, in an additional series of experiments, the first layer of positively charged gelatin A was deposited and, then, EGCG/(GelB/EGCG)$_3$ shell was formed around it. The uneven distribution of TRITC fluorescence remained. The fact that the shells around the cores can be constructed using both gelatin A and gelatin B is in good agreement with the data obtained by QCM. The observed inconsistency of the (GelB/EGCG)$_4$ capsules appearance in CLSM images with QCM data can be explained by slightly different conditions of the assemblies. All QCM measurements were done with drying the whole film after adsorption of each layer. Under these conditions each new layer is adsorbed on the surface of an already compacted and dense film. Due to formation of hydrophobic gelatin/EGCG network re-hydration and swelling of such films in aqueous media is apparently low. On the contrary, the (gelatin/EGCG)$_4$ shells on the cores were assembled in “wet” conditions without any intermediate drying between adsorption step and the formed loose EGCG/gelatin structures are “soaked” with water. For positively charged gelatin A alternated with negatively charged EGCG (Fig. 5), both hydrophobic and electrostatic forces support the formation of uniform shells around the cores. But for negatively charged gelatin B, due to electrostatic repulsion between adsorbed protein and EGCG the hydrophobic...
material of the shell tends to arrange in a more thermodynamically preferred structure consisted of large and small hydrophobic aggregates.

3.5. (GelA/EGCG)₄ capsules

The (GelA/EGCG)₄ capsules obtained after core dissolution are very light and highly hydrophobic. They tend to reach a drop border and attach to unwetted glass or to each other. The average diameter of (GelA/EGCG)₄ capsules is close to the size of initial cores. One can see (Fig. 7) that the majority of the capsules are slightly deformed, probably due to high hydrophobicity of the wall material, up to ca. 20% of capsules are collapsed, broken, or have holes. None of the techniques tested allows to prepare freestanding GelB/EGCG capsules. We speculate here that properties of the used gelatins influence formation of stable uniform defect-free protein/polyphenol shells around cores and free-standing capsules in aqueous media. Such coatings or microcapsules apparently can not be obtained if the major type of interaction between polyphenol and protein components in a system is hydrogen bonding; electrostatic forces play essential role in stabilization of the systems in aqueous media.

According to the EDAX analysis, the atomic ratio of elements (C:O:Mn) in the capsule material is 94.19:5.70:0.11. With the Mn signal being on the level of background noise, the capsule material contains practically no traces of core material.

As one can see from SEM and ACM images (Fig. 8), the diameter of the (gelatin/EGCG)₄ capsules after drying is about 4–5 μm. Drying causes a collapse of the gelatin/EGCG capsules with formation of typical folders on capsule walls. The capsule walls consist of gelatin/EGCG aggregates with a diameter of 50–300 nm and a height of 5–7 nm. The thickness of two folded walls of a four bilayer GelA/EGCG capsule is 28–30 nm, the value corresponds to a gelatin/EGCG bilayer thickness of approximately 3.7 nm. This value is lower than that found for GelA/EGCG bilayers by QCM (8.5 ± 4.3 nm per layer, averaged for four first bilayers). The inconsistency can be explained by different experimental conditions of the assemblies. The films on quartz resonators were dried after each adsorbed layer, while capsule walls were assembled in “wet” conditions.

Both on SEM and AFM images of (GelA/EGCG)₄ capsules, a fraction of small nanoparticles separated from the capsules was observed (Fig. 8, indicated with a white arrow). We assume that
those are EGCG/gelatin aggregates formed due to slow partial dissolution of the capsule material. Protein/polyphenol binding is principally a reversible process, and a slow dissolution of protein/polyphenol complexes occurs while the conditions are changed [13–19].

Permeability of (GelA/EGCG)₄ capsule walls for FITC-labeled dextrans of different molecular weight was tested at pH 6.5 in water. Only unbroken unfolded capsules were counted. The ratio of fluorescence intensities in the inner volume of the capsules and in surrounding solution decreases with increasing dextran's MW (Fig. 9). A delayed interior fluorescence was observed for dextrans of all molecular weights tested as well as for low molecular weight FITC. At pH 6.5, similar results have been previously observed only for tannic acid/chitosan microcapsules [21]. Several other types of capsules, including those of tannic acid/polyelectrolyte [20] and polyallylamine hydrochloride/polystyrene sulfonate [23,24] are completely permeable at all pH for substances with molecular weight lower than several thousands. Only tannic acid/poly(diallyldimethylammonium chloride) capsules showed slightly delayed interior fluorescence in the case of dextran with molecular weight of 4000 at pH 6.5 [20]. Formed directly on the surface of compacted drug form or combined with template polyelectrolyte microcapsules, the GelA/EGCG shells due to size selective permeability can be used to control the release of encapsulated compounds over wider range of pH and molecular weights, and what is more, to introduce additional antioxidant properties due to polyphenol adsorption to the capsule walls.

In conclusion, LbL assembly of a natural polyphenol, (−)-epigallocatechin gallate, in alternation with proline-rich proteins, gelatins, has been demonstrated for the first time. The fact that both type A and type B gelatins, having different isoelectric points and being of different charge under experimental conditions, in alternation with EGCG form relatively bulk multilayers suggests that the interaction between the components is mainly hydrophobic. However, a contribution of electrostatic forces into the interaction between components seems to be necessary in order to obtain a stable defect-free protein/polyphenol coating around microcores or free-standing microcapsules in aqueous media, as it occurs for the GelA/EGCG system.

Since the EGCG content in the gelatin/polyphenol film material is rather high (up to 30% w/w), encapsulation of EGCG in LbL assembled films and microcapsules can be a perspective way to obtain new formulation of this cancer chemopreventive polyphenol for drug delivery applications.

Supporting information

The online version of this article contains additional supporting information.

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References