De Novo Design of Supercharged, Unfolded Protein Polymers and their Assembly into Supramolecular Aggregates


Materials

E. coli XL1-Blue competent cells were purchased from Stratagene (La Jolla, CA). The pUC19 cloning vector, restriction endonucleases, T4 DNA ligase (LC), Fast AP™ thermosensitive alkaline phosphatase (Fast AP), and GeneJET™ Plasmid Miniprep kit were purchased from Fermentas (St. Leon-Rot, Germany). Digested DNA fragments were purified using QIAquick® spin miniprep kits from QIAGEN, Inc. (Valencia, CA). The pET-25b(+) vector and E.coli BLR(DE3) competent cells were purchased from Novagen Inc. (Milwaukee, WI). Oligonucleotides were synthesized by biomers.net (Ulm, Germany). Bacto™ tryptone and BBL™ yeast extract were purchased from Becton, Dickinson and Co. (Sparks, MD). Potassium phosphate monobasic, potassium phosphate dibasic, sodium phosphate monobasic, sodium phosphate dibasic, sodium chloride, and glycerol were purchased from Merck KGaA (Darmstadt, Germany). Ampicillin and imidazole were purchased from Roth (Karlsruhe, Germany). Isopropyl ß-thiogalactopyranoside (IPTG) was purchased from Duchefa (Harlem, Netherlands). 3,5 dimethoxy-4-hydroxycinnamic acid and internal standards bovine serum albumin and trypsinogen were purchased from LaserBio Labs (Sophia-Antipolis, France). Poly(sodium 4-styrenesulfonate) (PSS, Mw ~70,000), poly(allylamine hydrochloride) (PAH, Mw ~56,000), poly(fluorescein isothiocyanate allylamine hydrochloride) (PAH-FITC, Mw ~56,000), poly-L-arginine (pARG, Mw> 70 kDa), dextran sulfate (DEXS, Mw ~10 kDa), poly-L-lysine hydrobromide (pLL, Mw 15,000 – 30,000 Da), calcium chloride dehydrate (CaCl₂), Sodium carbonate (Na₂CO₃) and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma-Aldrich. Alexa Fluor488, Alexa Fluor 594 carboxylic acid succinimidyl ester and amino dextran (Mw ~500,000) carboxylic acid succinimidyl ester were obtained from Molecular Probes (Invitrogen). All chemicals were used as received. Ultrapure water with a resistivity greater than 18.2 MΩ cm was used for all experiments.

Monomer gene synthesis and gene oligomerization

Construction of the monomer genes and subsequent multimerization were performed as described by Chilkoti and co-workers.[1] Briefly, a monomer gene was constructed from eight 5’-phosphorylated, PAGE-purified synthetic DNA oligonucleotides. For annealing, equimolar mixtures of the oligonucleotides in T4 DNA ligase buffer were heated to 94°C and then slowly cooled down to 4°C, yielding a double-stranded DNA cassette with EcoRI and HindIII compatible ends. pUC19 was digested with EcoRI and HindIII, dephosphorylated with Fast AP and run on a 1% agarose gel in TAE buffer (per 1L, 108 g Tris base, 57.1 mL glacial acetic acid, 0.05 m EDTA, pH 8.0). The vector band was cut out and purified using a spin column purification kit. The annealed oligonucleotides were ligated to the linearized vector. For transformation, 200 µL of chemically competent E.coli
XL1-Blue cells were combined with 5 µL of the ligation mixture and further treated according to the manufacturer’s protocol. Cells were spread on Lysogeni broth (LB) agar plates (for 1L, 10 g Bacto™ tryptone, 5 g BBL™ yeast extract, 5 g NaCl, 15 g agar) supplemented with 100 µg/mL ampicillin, and incubated over night at 37ºC. Colonies were picked and grown in 6 mL LB media (for 1L, 10 g Bacto™ tryptone, 5 g BBL™ yeast extract, 5 g NaCl) supplemented with 100 µg/mL ampicillin over night, and plasmids were isolated using the GeneJET Plasmid Miniprep kit. Positive clones were verified by plasmid digestion with EcoRI and HindIII and subsequent gel electrophoresis. The DNA sequence of putative inserts was further verified by DNA sequencing (SequenceXS, Leiden, The Netherlands).

Gene oligomerization was performed as described by Chilkoti and co-workers. Positive clones were verified by plasmid digestion with EcoRI and HindIII and subsequent gel electrophoresis. The DNA sequences of putative inserts were further verified by DNA sequencing (SequenceXS, Leiden, The Netherlands). Gene sequences and respective amino acid sequences of K48 and E57 are shown in figure S1. As the recognition sites of the restriction enzymes PflMI and BglI have to be preserved, a valine residue instead of a lysine or glutamic acid residue is incorporated with every step of oligomerization.

a) Gene sequence of ELP K48

<table>
<thead>
<tr>
<th>Gene Oligomerization</th>
<th>EcoRI</th>
<th>PflMI</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCG GGC AAA GGT GTT CCT GTT AAA GGT GTG CCG GTG AAA GGT GTG CCG GGC AAA GGT</td>
<td>P G K G V P G K G V P G K G V P G K G V</td>
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<tr>
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</tr>
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</tr>
<tr>
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<tr>
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</tr>
<tr>
<td>GTG CCG GGC GTG GTT CCG GGC AAA GGT GTT CCG GTG AAA GGT GTG CCG GGC AAA GGT</td>
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<tr>
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<tr>
<td>GTG CCG GGC AAA GGT GTT CCT GTG AAA GGT GTG CCG GTG AAA GGT GTG CCG GTG AAA</td>
<td>V P G K G V P G K G V P G K G V P G K G V</td>
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BglI HindIII

GGG CTG GAA TA
b) Gene sequence of ELP E57

<table>
<thead>
<tr>
<th>Gene Sequence</th>
<th>Enzymes</th>
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<tr>
<td><strong>EcoRI</strong></td>
<td>AATT CAT ATG GCC CAC GCC GTG GTT CCG GCC GAA GGT GTT CCG GGT GAA GGT GTG</td>
</tr>
<tr>
<td><strong>PflMI</strong></td>
<td>CCG GCC GAA GGT GTT CCT GTG GAA GGT GTG CCG GCC CCG GCC GAA GGT</td>
</tr>
<tr>
<td><strong>NdeI</strong></td>
<td>GGT CCG GCC GAA GGT GTG CCG GCC GAA GGT GTT CCG GCC GAA GGT GTG</td>
</tr>
<tr>
<td><strong>BglII</strong></td>
<td>GGT GTT CCG GCC GAA GGT GTG CCG GCC GAA GGT GTT CCG GCC GAA GGT GTG</td>
</tr>
</tbody>
</table>

Figure S1. Genes and corresponding polypeptide sequences of (a) ELP K48 (lysine variant) and (b) ELP E57 (glutamic acid variant). Recognition sites for the restriction enzymes EcoRI, PflMI, BgII, and HindIII are underlined.

Expression vector construction

The expression vector pET 25b(+) (Novagen) was modified by cassette mutagenesis as described by Chilkoti and co-workers.[1] The DNA sequence spanning NdeI to EcoRI was exchanged for a
sequence which incorporates a unique Sfi I recognition site and which encodes for an affinity tag consisting of six histidine residues (Fig. S2). The modified pET 25b(+) expression vector was digested with SfiI, dephosphorylated and purified using a microcentrifuge spin column kit. The ELP gene was excised from the pUC19 vector by digestion with PflMI and BglI, and the excised gene was purified by agarose gel extraction following gel electrophoresis. The linearized vector and ELP-encoding gene were ligated, transformed into XL1-Blue cells, and screened as described above.

**Insert sequence of modified expression vector pET-25b(+) SfiI-H6**

<table>
<thead>
<tr>
<th>NdeI</th>
<th>SfiI</th>
<th>EcoRI</th>
</tr>
</thead>
<tbody>
<tr>
<td>TATG GGC GCG GGG GGG GGC</td>
<td>TGG CCG CAC CAC CAC CAC TGA TAA GAA TT</td>
<td></td>
</tr>
<tr>
<td>M G A G P G W P H H H H H H</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure S2. Sequence inserted into pET-25b(+) between recognition sites NdeI and EcoRI. The modified pET-25b(+)-SfiI-H6 vector contains a unique SfiI recognition site to insert ELP genes into the vector, and sequence encoding for a hexa-histidine (H6) tag at the C-terminus of the expressed protein for affinity purification.

**Protein expression and purification**

*E. coli* BLR (DE3) cells (Novagen) were transformed with the pET expression vectors containing the respective ELP genes. For protein production, Terrific Broth medium (for 1L, 12 g tryptone and 24 g yeast extract) enriched with phosphate buffer (for 1L, 2.31 g potassium phosphate monobasic and 12.54 g potassium phosphate dibasic) and glycerol (4 mL per 1L TB), and supplemented with 100 µg/mL ampicillin was inoculated with an overnight starter culture to an initial OD$_{600}$ of 0.1 and incubated at 37°C with orbital agitation at 250 rpm until OD$_{600}$ reached 0.7. Protein production was induced by addition of IPTG to a final concentration of 1 mM. Cultures were then continued for an additional 4 h post-induction. Cells were subsequently harvested by centrifugation (7,000 x g, 20 min, 4°C), resuspended in lysis buffer (50 mM sodium phosphate buffer, pH 8.0, 300 mM NaCl, 20 mM imidazole) to an OD$_{600}$ of 100 and disrupted with a French Press. Cell debris was removed by centrifugation (40,000 x g, 90 min, 4°C). Proteins were purified from the supernatant under native conditions by Ni-sepharose chromatography (GE Healthcare). Product-containing fractions were pooled and dialyzed against Ultrapure water (>18 MΩ). K48 was further purified by affinity chromatography using a Heparin HP column (GE Healthcare) and protein-containing fractions were dialyzed against Ultrapure water (>18 MΩ). Purified proteins were stored at -20°C or lyophilized and stored at room temperature until further use.

**Protein characterization**

The concentrations of the purified ELPs were determined by measuring absorbance at 280 nm using a spectrophotometer (NanoDrop™, Thermo Scientific). Protein purity was analyzed by PAGE (polyacrylamide) gel electrophoresis on a 4-12% NuPAGE® Bis-Tris gel (Invitrogen) and subsequent staining of the gel with SimplyBlue™ SafeStain (Invitrogen). Furthermore, purified proteins were characterized by SDS-PAGE (SDS - sodium dodecyl sulfate) on a 12% polyacrylamide gel according to Laemmli[2] and subsequent copper (II) chloride staining as reported by Lee and coworkers.[3] Mass spectrometric analysis was performed using a 4800 Plus MALDI-TOF/TOF Analyzer (Applied Biosystems, Foster City, CA, USA). The samples were prepared in a recrystallized 3.5 dimethoxy-4-hydroxycinnamic acid matrix with the calibration standards bovine serum albumin (MW = 66,429.9) for K48 and trypsinogen (MW = 23980.9) for E57. Mass spectra
were recorded in positive ion mode with the 4000 Series Explorer Software, version 3.0 (Applied Biosystems, Foster City, CA, USA). The data were analyzed in Data Explorer, version 4.9 (Applied Biosystems, Foster City, CA, USA). The masses determined by MALDI-TOF were 24,105 +/- 50 Da for K48 and 28,967 +/- 50 Da for E57, which is in excellent agreement with the calculated masses of 24,150.7 and 28,970.1 Da, respectively.

![MALDI-TOF mass spectra of supercharged elastin-like proteins K48 and E57. a) Mass spectrum of K48 (m/z is 24,104.6) with internal standard bovine serum albumin (BSA; m/z is 22,135.5 for BSA +3H and m/z is 33,215.3 for BSA +2H). b) Mass spectrum of E57 (m/z is 28,967.1) with internal standard trypsinogen (m/z is 23,982.0). I = absolute intensity.](image1)

**Figure S3.** MALDI-TOF mass spectra of supercharged elastin-like proteins K48 and E57. a) Mass spectrum of K48 (m/z is 24,104.6) with internal standard bovine serum albumin (BSA; m/z is 22,135.5 for BSA +3H and m/z is 33,215.3 for BSA +2H). b) Mass spectrum of E57 (m/z is 28,967.1) with internal standard trypsinogen (m/z is 23,982.0). I = absolute intensity.

**Analysis of secondary structure**

Circular dicroism (CD) spectra were recorded using a Jasco-815 spectropolarimeter (Jasco, Japan). Measurements were carried out at room temperature with a cell path length of 1 mm. The polypeptide concentrations in Ultrapure water were 10 and 5 µM for K48 and E57, respectively. The CD spectra of both polypeptides showed a smaller trough at around 220 nm and a larger trough at around 200 nm (Fig. S4). This spectral behavior is usually interpreted to represent largely random coil structure with some contribution of α-helical segments.\[4, 5\]

![Circular dicroism (CD) spectra of aqueous solutions of ELPs K48 (10 µM) and E57 (5µM). Data represent averages of 25 scans.](image2)

**Fig. S4.** Circular dicroism (CD) spectra of aqueous solutions of ELPs K48 (10 µM) and E57 (5µM). Data represent averages of 25 scans.
LCST behavior analysis

To characterize the ELP inverse temperature transition, the OD$_{350}$ of K48 and E57 ELPs in ultrapure water at a concentration of 57 and 24 µM, respectively, were measured as a function of temperature on a Jasco V630 spectrophotometer. Measurements were performed between 20 and 90ºC by increasing the temperature every 10 min in 5ºC increments. No significant increase in the OD$_{350}$ values for either of the ELP variants could be observed at any temperature measured. OD$_{350}$ values were below 0.1 at all times. This finding is in line with published data, where the incorporation of increasing numbers of lysine or glutamic acid residues in ELPs led to an increase in the lower critical solution temperature (LCST) at neutral pH values.\[6, 7\]

Labelling of proteins

Alexa Fluor® 488 sulfodichlorophenol ester (AF488) was purchased from Molecular Probes (Invitrogen) and dissolved in DMF to a concentration of 10 mg/mL. To 3.15 mg of K48 in 0.1 m sodium carbonate buffer, pH 8.6, an equimolar amount of AF488 was added under vigorous stirring. After incubation for 2 h at room temperature under vigorous stirring, hydroxyamine solution (pH 8.6) was added to a final concentration of 0.14 m and incubated for additional 90 min at room temperature. Uncoupled dye was removed by size exclusion using an illustra NAP™-25 column (GE Healthcare) and 0.1 m sodium carbonate buffer (pH 8.6) as equilibration and elution buffer. Protein-containing fractions were pooled and dialyzed (cut-off 500 Da) against Ultrapure water (>18 MΩ). Protein concentration was determined using the following equation:

\[
c\ [\text{mg/mL}] = (A_{280} - 0.11 \times A_{495}) \times \text{MW}_{\text{K48}}
\]

where $A_{280}$ and $A_{495}$ are the absorbance values at 280 and 495 nm, respectively, and MW$_{\text{K48}}$ is the molecular weight of K48. The labelled protein (K48AF488) was lyophilized and stored at room temperature until further use.

Cytotoxicity assay of positively charged polyelectrolytes (PEs)

NIH/3T3 embryonic fibroblasts were seeded in a 96-well-plate (Greiner by Sigma-Aldrich) at a cell density of $10^4$ cells/well in 100 µL growth medium (DMEM-F12 Ham’s basal medium supplemented with 10% calf serum, 1% L-glutamine and 1% penicilline/streptomycine whereby all material were provided by Sigma-Aldrich). The next day, the cells were incubated with the PEs under investigation (i.e. PLL, PAH, pARG, and K48) for 24h. A starting concentration of 1 mg/mL was used for each PE and consecutively halved until a final concentration of 6.1 x $10^{-5}$ mg/mL. All concentrations were done in duplicate. Cells that were not treated with any PE served as a positive control for viability. After 24 h, the cells were washed once with PBS, 100 µL of a 10% Resazurin (TOX-8 kit from Sigma-Aldrich) solution (in growth medium) were added to each well and incubated for 3 h at 37ºC and 5% CO$_2$. Resazurin is a blue, non-fluorescent sodium salt, which is converted to resorufin by metabolically active cells. Resorufin is a pink, fluorescent sodium salt that accumulates outside the cells. This reduction process requires functional mitochondrial activity which is inactivated immediately after cell death. Fluorescence spectra were measured using a 96-microwell plate reader connected to a Fluorolog® spectrofluorometer (Jovin Yvon) at an excitation wavelength of 560 nm. The emission was recorded in the range of 572-650 nm with 1 nm resolution and a slit of 5 nm. Firstly, the mean of the intensities of the emission spectra of the duplicates was calculated and then, the maximum intensity values found in the range 578-585 nm were also averaged. The mean background signal (640-650 nm) was subtracted from the mean maximum emission values and subsequently normalized with the maximum fluorescence value obtained. The maximum fluorescence value corresponded not always to the untreated cells, probably due to the formation of hydroresorufin, a transparent nonfluorescent product which is formed upon further reduction of...
resorufin by viable cells. The experiments were repeated three times for each PE. The means of the normalized fluorescence intensity values of the three experiments (I/Imax) were plotted against the different concentrations of the PEs. A sigmoidal distribution was obtained and fitted as a function of a logistic dose response curve which enables us calculating the PE concentration yielding 50% cell death (LD50).

**Charges of positive and negative polyelectrolytes (PEs)**

The main driving force in alternate LBL assembly of multilayer capsules is the electrostatic interaction between oppositely charged species. Hence, the charges per molecule of the two supercharged unfolded proteins K48 and E57, employed as component layer of ELPs-derived capsules, was calculated and compared to the number of charges per molecule of the other PEs used for the synthesis of capsules controls (i.e. PAH, pARG, PSS and DEXS) (Table 1). As expected, all the control PEs have a higher number of charges per molecule than the ELPs except for DEXS, which has around 27 charges per molecule. The µmol charges per 1 mg are also higher for all of the control PEs than for the ELPs.

<table>
<thead>
<tr>
<th>PE</th>
<th>full name</th>
<th>MW (average) [Da]</th>
<th>charges/molecule at pH 7.0</th>
<th>µmol charges in 1 mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>E57</td>
<td>ELP-glutamic acid</td>
<td>28,970</td>
<td>57</td>
<td>1.97</td>
</tr>
<tr>
<td>K48</td>
<td>ELP-lysine</td>
<td>24,150</td>
<td>48</td>
<td>1.99</td>
</tr>
<tr>
<td>DEXS</td>
<td>Dextran sulfate sodium salt from <em>Leuconostoc</em> spp.</td>
<td>6,500-10,000</td>
<td>27.79</td>
<td>3.37</td>
</tr>
<tr>
<td>pARG</td>
<td>Poly-L-arginine hydrochloride</td>
<td>70,000</td>
<td>498</td>
<td>7.11</td>
</tr>
<tr>
<td>PSS</td>
<td>Poly(sodium 4-styrenesulfonate)</td>
<td>70,000</td>
<td>339</td>
<td>4.84</td>
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<tr>
<td>PAH</td>
<td>Poly(allylamine hydrochloride)</td>
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<td>605</td>
<td>10.8</td>
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<tr>
<td>pLL</td>
<td>poly-L-lysine hydrobromide</td>
<td>15,000-30,000</td>
<td>108</td>
<td>4.8</td>
</tr>
</tbody>
</table>

Table 1: Calculation of the charges per molecule and µmol charge per 1 mg of each PE (E57, K48, DEXS, pARG, PSS and PAH).

**Capsule preparation**

The negatively charged E57 and the positively charged K48 ELPs were alternatively assembled onto inorganic calcium carbonate (CaCO$_3$) microparticle templates for the preparation of hollow polypeptide microcapsules (Scheme S1). CaCO$_3$-based cores were used because compared to organic templates they allow for the synthesis of capsules under mild conditions, as the CaCO$_3$ core material can be easily removed by complexation with ethylenediaminetetraacetic acid (EDTA) buffer. Additionally, the encapsulation of biomacromolecules can be performed directly during the synthesis of the CaCO$_3$ microparticles by entrapping the cargo within the sacrificial template particles, owing to their high porosity. As control samples, capsules made of degradable and non-degradable polyelectrolytes were produced. Degradable capsules, susceptible to enzymatic degradation, were composed of dextran sulfate (DEXS) as the polyanion and of poly-L-arginine (pARG) as the polycation, non-degradable capsules were made of poly(sodium 4-
styrenesulfonate) (PSS) as the polyanion and of polyallylamine hydrochloride (PAH) as the polycation.

**Scheme S1.** Schematic representation of polyelectrolyte capsule fabrication by layer-by-layer (LbL) assembly. (i) Spherical CaCO$_3$ microparticles are fabricated by precipitation from supersaturated CaCl$_2$ and Na$_2$CO$_3$ solutions. (ii–iii) Oppositely charged polymer layers are consecutively adsorbed around the spherical templates by electrostatic attractions. (iv) The original template is removed by dissolution to obtain a capsule with an empty cavity. Only few layers of polyelectrolyte are shown, for sake of clarity. Capsules are not drawn to scale.

**Preparation of CaCO$_3$ microparticles**

For each capsule system, CaCO$_3$ microparticles were precipitated from solutions of calcium chloride and sodium carbonate under vigorous stirring.$^{[10]}$ Briefly, equal volumes (0.615 mL) of aqueous CaCl$_2$ and Na$_2$CO$_3$ solutions (0.33 M) were mixed in the presence of 5 mg/mL 500 kDa dextran and thoroughly agitated on a magnetic stirrer for 30 s at room temperature. After the agitation, the mixture was left without stirring for 4 min at room temperature. During this time precipitation of CaCO$_3$ occurs and spherical CaCO$_3$ particles with an average diameter ranging from 2.5-3.5 µm are formed. Dextran is integrated in the cores.$^{[13]}$ Subsequently, the precipitate was separated from the supernatant by centrifugation (6000x g, 6 s) and washed three times with pure water to remove unreacted species. In the last step, the particles were washed with acetone and air-dried. We incorporated dextran in the CaCO$_3$ cores, as dissolution of the cores including dextran by addition of EDTA was faster than that of cores without dextran. Naturally, in this way dextran remains in the capsule cavities after core dissolution. The whole powder obtained from one synthesis (about 20 mg) was employed for the LbL coating.

**Fabrication of multilayer capsules**

The resulting spherical cores were coated by sequential incubation of the particles in the corresponding polyanion and polycation solutions. Three different types of microcapsules made of different layer constituents were prepared by sequential adsorption of negatively charged and positively charged species on CaCO$_3$ microparticles (~20 mg per samples) to give the following shell architectures comprising 11 layers in total:

- **PSS/PAH - capsules:** (PSS/PAH)$_3$(PSS/PAH$_F$ITC)(PSS/PAH)PSS,
- **DEXS/pARG - capsules:** (DEXS/pARG)$_3$(DEXS/pARG$_{AF488}$)(DEXS/pARG)DEXS,
The adsorption of polyelectrolytes PSS, PAH, PAH\textsubscript{FITC} and DEXS was conducted in 2 mg/mL solutions in 0.5 m NaCl, whereas the polyelectrolyte pARG and the positively charged and negatively charged proteins (K48 and E57, respectively) were suspended in 1 mg/mL solutions in 0.5 m NaCl. The pH of the polyelectrolyte solutions was adjusted to 6.5 by addition of NaOH, whereas the pH of the protein solutions was maintained neutral (∼7.2-7.6). The adsorbing protocol started with the negatively charged polymer (PSS or DEXS). PAH\textsubscript{FITC} (obtained from Sigma) and PAH\textsubscript{AF488} (obtained by reacting NHS-ester modified Alexa488 to the amino groups of PAH or pARG) were used instead of non-labeled polycation for the eighth layer of the multilayer polymer shell, so that the capsules had a green emitting dye label in their walls. Similarly, K48\textsubscript{AF488} was used for the tenth layer of the protein capsules to label the capsule walls. After addition of each charged species, samples were continuously shaken for 12 min. The coated particles were then centrifuged at 6000x g for 6 s and the supernatant containing unabsorbed species was removed. This procedure was repeated three times after each absorption step. After each cycle the CaCO\textsubscript{3} suspension was resuspended with ultrasound pulses to prevent aggregation. At the end eleven layers were deposited for each capsule type, starting from the polyanion. We want to point out that in the case of the protein capsules the first two layers were DEXS and pARG in order to mechanically stabilize the capsules. As the capsule cavities include dextran, the first layer of dextran sulphate has the same constituency as the interior of the capsule cavity. Poly-L-arginine is a polypeptide and thus similar in nature to the following layers of supercharged proteins. Both DEXS and pARG are biodegradable.\textsuperscript{[12]} After assembly of the capsule walls by LbL deposition the CaCO\textsubscript{3} core was removed by complexation with EDTA. Coated CaCO\textsubscript{3} particles were shaken for 2 min with 1 mL of an EDTA solution (0.2 M, pH 5), followed by centrifugation and redispersion in 1 mL of a fresh EDTA solution (0.2 M, pH 7). The thus obtained hollow microcapsules with some dextran in their cavities were washed five times with pure water with centrifugation at 1000 x g for 8 min. The microcapsules were finally stored as suspension in water at 4°C.

**Capsule characterization**

**Confocal laser scanning microscopy (CLSM)**

Fluorescent images were taken by a confocal microscope (LSM 510 META, Zeiss). The excitation wavelength was 488 nm. Samples were observed through a 100X/1.45 NA oil-immersion PLAN-FLUOAR objective. Capsules labeled with FITC and Alexa488 fluorescence were studied with the Ar/Kr laser 488 nm. A 20 µL drop sample was placed onto a cover glass and imaged in liquid.

The typical morphologies of core-shell microparticles after LbL assembly with the same number of layers but with different layer components are presented in Figure S5. In the three systems investigated the diameter of the microparticles was found to be in the range of 2-2.5 µm. As shown in the fluorescence channels, the fluorescence signal corresponding to the labeled layers, which had been added as tenth layer in the ELP capsules and as eighth layer in the biodegradable and non-degradable capsules, was detected only from the walls. This indicates that the layers were efficiently adsorbed around the spherical templates during the LbL assembly. As expected, the CaCO\textsubscript{3} cores were clearly visible in the corresponding transmission channels. By overlaying the fluorescent and transmission channels, the two compartments of the capsules, walls and cavities, were distinguished.

E57/K48 - capsules: (DEXS/pARG)(E57/K48)\textsubscript{3}(E57/K48\textsubscript{AF488})E57.
Figure S5. CLSM images of (a) (DEXS/pARG)(E57/K48)$_2$(E57/K48$_{AF488}$)E57, (b) (DEXS/pARG)$_3$(DEXS/pARG$_{AF488}$)(DEXS/pARG)DEXS and (c) (PSS/PAH)$_3$(PSS/PAH$_{FITC}$)(PSS/PAH)PSS capsules before core removal. The capsule walls were labeled with AF488 (a, b) and FITC (c). Left panels: Fluorescence images of green emitting dyes. Central panels: optical transmission images. Right panels: corresponding overlay of both fluorescence and transmission channels. The fluorescence signal coming from the capsule walls can be seen whereas the spherical shape of CaCO$_3$ porous cores can be observed in the corresponding transmission images. Scale bars represent 1 µm.

In a previous experiment, protein capsules assembled by using E78 as first layer showed the diffusion of the fluorescent layer K48$_{AF488}$ inside the capsule cavity during the LbL steps (Fig. S6). Moreover after core removal, no spherical capsules were detected under fluorescent microscopy, thus confirming that both the E57 and the K48 polypeptides were mostly localized as complexes inside the CaCO$_3$ cores instead of alternately depositing at the template surface. It is worth noting that CaCO$_3$ microparticles are characterized by a high porosity which pores ranging from 20 to 60 nm. This allows small molecules with a size of several nanometers to penetrate inside the templates during the LbL assembly. Thus, in order to prevent the diffusion of the ELP inside the cavities, we decided to start to build up the ELP multilayer shell after adsorption of one biodegradable bi-layer made up of (DEXS/pARG) polymers. This results in the final structure of E57/K48 - capsules: (DEXS/pARG)(E57/K48)$_3$(E57/K48$_{AF488}$)E57.
Figure S6. Fluorescence images of capsules consisting of 8-layers of (K48/E57)$_3$(K48$_{AF488}$/E57) ELP polypeptides. CaCO$_3$ core-shell microparticles before (a-b) and after (c-d) core removal. Fluorescence signal from the capsules cavities indicated the diffusion of the K48$_{AF488}$ polypeptide inside the capsule interior during the LbL assembly (a-b). After core removal, no spherical capsules were visible under the fluorescence microscope (c-d), whereas a fluorescent carpet layer and small aggregates of particles were observed due to the release of the K48$_{AF488}$ out of the microparticles following the core dissolution. This confirmed that the E57 and K48 polypeptides were mostly localized inside the CaCO$_3$ cores instead of depositing on the wall. Scale bars represent 2 µm.

After following the core removal procedure, CLSM pictures were taken of the resulting capsules (Figures S7 and S8). Notably, the fluorescence signal in the ELP capsules was found to be still confined to the walls even after core removal, showing that the integrity of the multilayer polypeptide shell (DEXs/pARG)(E76/K48)$_3$(E76/K48$_{AF488}$)E76 was not affected during the core dissolution. Instead, in some of the biodegradable capsules a slight diffusion of the labeled polymer pARG$_{AF488}$ inside the cavities was observed, owing to the above mentioned porosity of the CaCO$_3$ cores. The absence of the cores was clearly detected in each capsule type sample, as shown in the transmission channels.
Figure S7. CLSM images of (a) (DEXS/pARG)(E57/K48)3(E57/K48AF488)E57, (b) (DEXS/pARG)3(DEXS/pARGAF488)(DEXS/pARG)DEXS and (c) (PSS/PAH)3(PSS/PAHFITC)(PSS/PAH)PSS single capsule after core removal. The capsule walls were labeled with AF488 (a, b) and FITC (c). The absence of the CaCO₃ cores can be clearly observed in the transmission images. Inset: CLSM images of several capsules showing spherical and intact capsules after core removal. Scale bars represent 1 µm.

Figure S8. Three-dimensional CLSM image (Top row) and corresponding cross-section profiles (Bottom row) of single capsules after core removal. The graphs in the bottom show the intensity of the fluorescence along the red lines traced on the single capsule. The peaks are due to emission from the AF488 (a and b) and FITC (c) fluorophores on the capsule walls.

The efficient assembly of the protein layers was confirmed by the following control experiment: CaCO₃ particles were coated with 1 bi-layer of DEXS/pARGAF488 and analyzed by CLSM before and after core removal (Figure S9). Before core removal, the typical morphology of capsules with a
green-fluorescent labeled wall was observed (Figure S9, top row) (pARG labeled with the dye AF488 was used enabling characterization by CLSM). After core removal, no capsules were observed in the bulk solution indicating that capsules made of only 1 bi-layer are not stable against the dissolution conditions. The core removal treatment with EDTA solution resulted in dismantling of the (DEXS/pARG) bi-layer shell, which showed fluorescence only in the bulk solution (Figure S9, bottom row). In contrast, the multilayers of the (DEXS/pARG)(E57/K48)_3(E57/K48AF488)E57-coated particles remained intact after exposure to EDTA (Figure S7). These data confirmed that the protein-based capsules prepared by LbL assembly of E57/K48 onto 1 bi-layer of (DEXS/pARG) were actually made of alternately assembly of the protein layers with the fluorescently labeled K48AF488 as second-last layer.

![Figure S9](image-url)

**Figure S9.** CLSM images of 1 bi-layer (DEXS/pARG<sub>AF488</sub>) capsules before (top row) and after (bottom row) core removal. AF488-labeled pARG was used as fluorescent layer. Before core removal, CaCO<sub>3</sub> cores can be clearly observed in the transmission channel, whereas the green signal indicates the presence of the pARG<sub>AF488</sub> layer only in the wall. After core removal, no capsules are found out in the bulk solution, indicating the disassembly of the bi-layer wall during the core decomposition. Scale bar represents 5 µm.

### Capsules permeability

In order to compare the permeability behavior of the protein-based capsules with the control capsules based on DEXS/pArg and PSS/PAH layers, dextran 500 kDa, labeled with AF594, was entrapped inside the cavities during the synthesis of the CaCO<sub>3</sub> cores. Figure S10 shows LSM images of the capsules before and after core removal. The wall of each capsule system was labeled with AF488. After core removal the diffusion of the dextran across the wall was observed in the (E57/K48) capsules whereas no diffusion was observed across the wall of control capsules. These data suggested the existence of large pores in the wall of supercharged protein-based capsules, as confirmed by further SEM and TEM analysis.
Figure S10. CLSM images of 11-layer (a) (E57/K48), (b) (DEXS/pARG) and (c) (PSS/PAH) capsules before and after core removal. The capsule cavities were loaded with 500 kDa dextran labeled with AF543, the capsule walls were labeled with AF488 (a, b) and FITC (c). The presence and the absence of the CaCO$_3$ cores can be clearly observed in the transmission images of each sample. After core removal the diffusion of the dextran outside was observed for the (E57/K48) capsules suggesting a higher porosity of the wall compared to the control capsules. Scale bars represent 5 µm.

Electron Microscopy

Protein capsules were then analyzed by Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) to gain deeper insight into the structure and morphology of the multilayer polypeptide wall, both before and after core removal, and to compare their properties to the control capsules. SEM micrographs were conducted with a JEOL JSM-7500F SEM at an operation voltage of 2.00 kV. A 10 µl drop sample was placed onto a cover glass, dried at room temperature, and sputtered with a platinum layer under vacuum for 90 s. TEM were recorded by using a JEOL 3010 TEM operating at an accelerating voltage of 300 kV. A 10 µl drop sample was placed on a Formvar®/carbon coated TEM-grid (300 Mesh 3.05 mm Copper, Plano GmBH) and dried at room temperature before imaging.

Figure S11 shows the SEM images corresponding to the core-shell microparticles previously presented in Figure S6. The images in the bottom row show a magnified area of the wall of the particles reported in the top row in which the surface texture of the particles can be appreciated. In general, protein-coated cores and (DEXS/pARG)-coated cores were characterized by a thick surface, suggesting that the polymers were densely packed into the multilayer shells. On the contrary, in the case of capsules made up of (PSS/PAH) polyelectrolytes, a highly porous surface was observed. This might be explained by the adsorption of the polyelectrolytes onto the very rough surface of the CaCO$_3$ microparticles which results in the formation of a very porous polyelectrolyte network of the
However, after dissolution of the templates, a porous network with clear holes was observed in the protein capsules sample (Fig. S12). These capsules correspond to the ones shown in Figure S7. A thick wall and a porous-like wall were detected in the biodegradable and non-degradable capsules.

**Figure S11.** SEM images of CaCO$_3$ cores after coating (a) (DEXS/pARG)$(E57/K48)_3$$(E57/K48_{AF488})E57$, (b) (DEXS/pARG)$_3$(DEXS/pARG$_{AF488}$)$(DEXS/pARG)DEXS$ and (c) (PSS/PAH)$_3$(PSS/PAH$_{FITC}$)$(PSS/PAH)PSS$. The typical spherical, porous-like structure of capsules templated on CaCO$_3$ cores can be observed. Scale bars represent 1 µm (top row), 100 nm (bottom row).

**Figure S12.** SEM images of (a) (DEXS/pARG)$(E57/K48)_3$$(E57/K48_{AF488})E57$, (b) (DEXS/pARG)$_3$(DEXS/pARG$_{AF488}$)$(DEXS/pARG)DEXS$ and (c) (PSS/PAH)$_3$(PSS/PAH$_{FITC}$)$(PSS/PAH)PSS$ capsules after core removal. Capsules collapse after core removal indicating the absence of the cores in their cavities. Scale bars represent 1 µm (top row), 100 nm (bottom row).

In line with the LSM and SEM data the walls of the protein capsules investigated under TEM were found to be more porous than biodegradable and non-degradable capsules (Fig. S13), indicating that the two investigated polypeptides, E57 and K48, formed thinner shells during the LbL assembly. Nonetheless we would like to point out that the porosity, and thus the permeability, of the protein capsules could be reduced by increasing the number of layers employed to grow the multilayer wall or by cross-linking the proteins after their adsorption onto the sacrificial core surfaces (i.e., by using glutaraldehyde as a cross-linker agent). Finally, the use of unfolded, supercharged proteins with higher number of charges per molecule (see Table 1) might be taken into account to build up a multilayer shell with stronger electrostatic attractions between each component layer.
Figure S13. TEM images of (a) (DEXS/pARG)(E57/K48)E57, (b) (DEXS/pARG)3(DEXS/pARGAF488)(DEXS/pARG)DEXS and (c) (PSS/PAH)3(PSS/PAHFITC)(PSS/PAH)PSS capsules after core removal. Capsules collapse after core removal indicating the absence of the cores in their cavities.

References