Encapsulated Oligonucleotides

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Degradable Polyelectrolyte Capsules Filled with Oligonucleotide Sequences**

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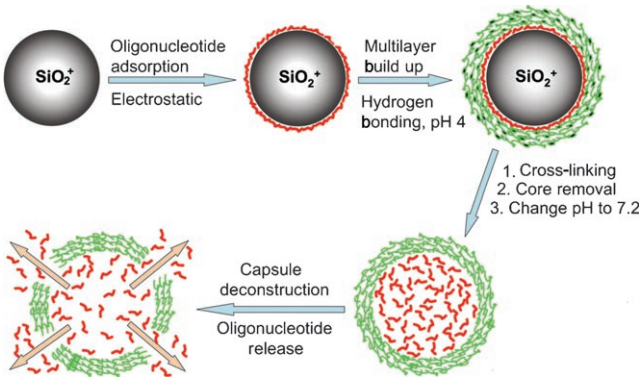
Hollow polyelectrolyte capsules prepared by the layer-by-layer adsorption of polymers on sacrificial template particles[1] hold immense potential for drug delivery.[2] The capsules can be engineered with controlled sizes, composition, and functionality, and can be loaded with model therapeutics such as proteins[3,4] and low-molecular-weight drugs.[5] However, methods for loading such capsules with gene vaccines remain largely unexplored. Recent work has shown that DNA can be precipitated onto template particles with spermidine, coated with polyelectrolyte multilayers, and subsequently released into the capsule interior upon dissolution of the core particle.[6] Another approach is based on rehydration of capsules in a DNA-containing solution.[7] Although these earlier studies demonstrate the loading of large DNA molecules (e.g., calf-thymus DNA, DNA from herring testes), encapsulation of short oligonucleotides, such as small-interfering (si)RNA, is of particular importance as these molecules are highly susceptible to degradation and therefore need protection during storage and cellular delivery.[8]

Herein, we report a polycation-free encapsulation method to obtain high concentrations of uncomplexed, short oligonucleotide chains confined within monodisperse, degradable microcapsules. The encapsulation method exploits amine-functionalized silica (SiO_2^+) particles to adsorb oligonucleotides, followed by the assembly of thiol-functionalized poly(methacrylic acid) (PMA_{SH}) and poly(vinylpyrrolidone) (PVPON) multilayers.[9] Removal of the template particles produces degradable capsules filled with oligonucleotides (Scheme 1). The key advantages of this method include: 1) the ability to attain high loadings of oligonucleotides ($>10^4$ chains per capsule); 2) quantitative incorporation of oligonucleotides from the starting solution into the final formulation with more than 90% of the capsules filled with DNA; 3) avoiding the use of mechanical forces, such as those typically applied in emulsion encapsulation processes, which can cause DNA degradation; and 4) the ability to release DNA under reducing conditions (as occurs in cells) that degrade the capsules.



Scheme 1. Schematic representation of the encapsulation of short oligonucleotide sequences within polyelectrolyte capsules.

The 1-μm diameter amine-functionalized SiO_2 particles (ζ-potential of 62 ± 4 mV at pH 4) were exposed to solutions of varying concentrations of carboxytetramethylrhodamine (TAMRA)-labeled oligonucleotides containing 15 repeating thymidine (T) residues followed by 15 repeating cytosine (C) residues, TAMRA-poly$T_{15}C_{15}$. The electrostatic interaction between the negatively charged poly$T_{15}C_{15}$ and the SiO_2^+ particles results in adsorption of the oligonucleotides. By monitoring the fluorescence intensity of the supernatant before and after oligonucleotide adsorption, the saturation coverage of the particles was determined as $\approx 0.3\ mg\,m^{-2}$ (see the Supporting Information). In all subsequent experiments, we chose a TAMRA-poly$T_{15}C_{15}$ surface coverage below the saturation limit ($0.24\ mg\,m^{-2}$).

The PMA_{SH}/PVPON multilayer build up was initiated by exposing the particles with preadsorbed poly$T_{15}C_{15}$ to a PMA_{SH} solution at pH 4. At this pH value, the carboxylic groups of PMA_{SH} are largely uncharged ($pK_a \approx 6.5$), and as a result only minor displacement of DNA from the particles into the bulk solution ($\approx 2\%$) was observed (see the Supporting Information). Adsorption of the second polymer, PVPON, as well as subsequent assembly of the multilayers proceed through hydrogen bonding of the polymers and results in no measurable loss of DNA, as determined by fluorescence spectroscopy. These data provide evidence that more than 95% of the initially introduced DNA was retained on the particles during deposition of the multilayers (up to 16 layers). We note that although the oligonucleotides electrostatically interact with the amine binding sites on the silica particles, PMA_{SH} adsorption is most likely a combination of electrostatic interaction with accessible positive charges on the particles and hydrogen bonding with the particles and/or the poly$T_{15}C_{15}$.

To analyze the multilayer build up, we used PMA_{SH} labeled with 0.1 wt% Alexa Fluor 488 maleimide dye (AF488) and flow cytometry as this technique allows rapid and quantitative analysis of the particles and simultaneous monitoring of fluorescence from multiple fluorophores (Figure 1). The PMA_{SH}/PVPON build up is reflected by an increase in AF488 fluorescence and a similar amount of PMA_{SH} is adsorbed on bare SiO_2^+ particles (curve 1) and

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Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

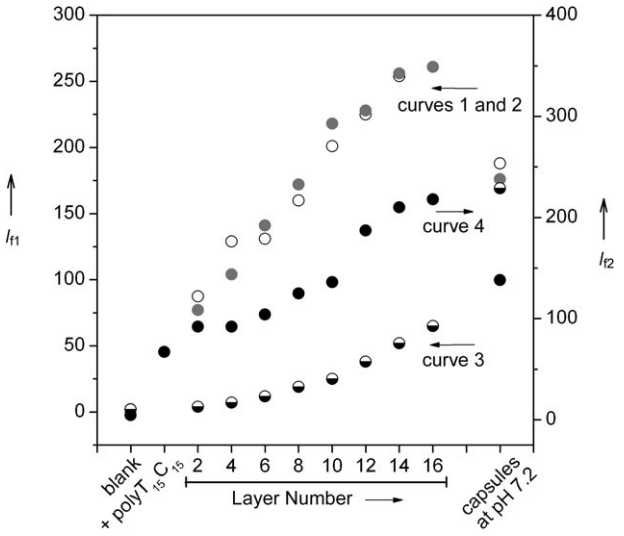

Figure 1. Flow cytometry data for the build up of PMA_{SH}/PVPON multilayers on 1 μm SiO_2^+ particles. Multilayer build up in the absence (curve 1) and presence (curve 2) of unlabeled $polyT_{15}C_{15}$ on SiO_2^+ particles, monitored through the fluorescence of the AF488 label (I_{f1}) on PMA_{SH}. Multilayer build up on particles with preadsorbed TAMRA-$polyT_{15}C_{15}$, monitored through the fluorescence of AF488 (I_{f1}) on PMA_{SH} (curve 3) and TAMRA (I_{f2}) on $polyT_{15}C_{15}$ (curve 4). The measurements were taken after the deposition of each PVPON layer.

those precoated with $polyT_{15}C_{15}$ (curve 2), including the deposition of the first PMA_{SH} layer. In contrast, when TAMRA-labeled $polyT_{15}C_{15}$ was used, we observed only a minor increase in AF488 fluorescence (curve 3) and a pronounced increase in TAMRA fluorescence (curve 4). This is readily attributed to the resonance energy transfer from AF488 to TAMRA and provides evidence that the PMA_{SH}/PVPON multilayer build up proceeds on the oligonucleotide-covered particle surface.[10]

To obtain stable capsules, the PMA_{SH} thiol groups were converted into disulfide linkages with the use of chloramine T,[11] the template particles were removed by dissolution with aqueous hydrofluoric acid/ammonium fluoride at pH 5,[12] and the capsules were washed and incubated at pH 7.2 for at least 24 h. Fluorescence analysis of the capsules (Figure 1) showed that in all three cases, the green fluorescence of the capsules was similar, including the capsules with TAMRA-$polyT_{15}C_{15}$. The red fluorescence of the capsules (curve 4) decreased and was similar to the level observed for the capsules obtained by using PMA_{SH} samples without the AF488 label (data not shown). These data provide evidence for the separation of TAMRA and AF488 dyes to a distance greater than the Förster radius and reflect separation of the oligonucleotide from the capsule wall into the interior of the capsule, that is, formation of the capsules with encapsulated free oligonucleotides.

Flow cytometry analysis of the capsules revealed that more than 90% of the capsules were filled with the DNA oligonucleotides (see the Supporting Information). The capsules are stabilized by disulfide linkages at physiological pH and retained the oligonucleotides over at least 72 h. As negligible loss of DNA was observed from the particle surface during multilayer assembly, each capsule contains up to 4.5×10^4 copies of the oligonucleotide. Compared with the 1-μm diameter of the template particles, the cross-linked PMA_{SH}/PVPON capsules at pH 7.2 are ≈1.5 μm in diameter, and the concentration of oligonucleotide chains corresponds to 40 μM (1.3 mM of phosphate groups).

Confocal laser scanning microscopy was used to visualize the oligonucleotide-filled capsules (Figure 2). The images

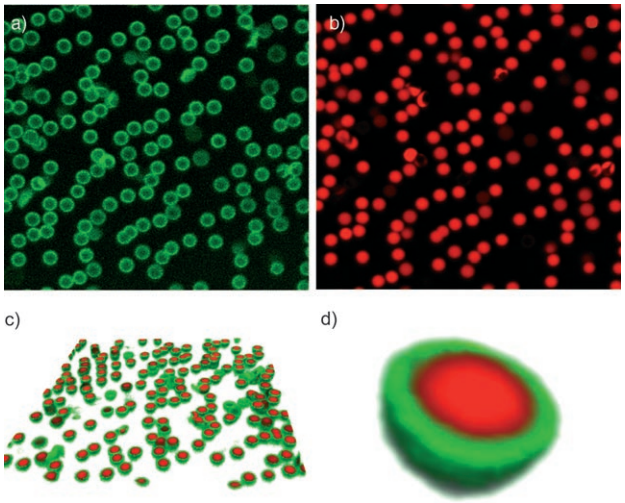

Figure 2. Confocal laser scanning microscopy images of 16-layer PMA_{SH}/PVPON capsules filled with $polyT_{15}C_{15}$ showing the fluorescence originating from the capsule walls owing to the PMA_{SH} labeled with AF488 (a) and the fluorescence of TAMRA-$polyT_{15}C_{15}$ (b), and 3D cross-section reconstruction images of the confocal data (c, d). The images (a–c) are 30×30 μm^2. The capsule in (d) is 1.5 μm.

provide proof of monodisperse capsules loaded with oligonucleotide sequences. The images show well-defined capsule walls (PMA_{SH} labeled with AF488, green, Figure 2 image a) and encapsulated DNA (TAMRA-$polyT_{15}C_{15}$, red, Figure 2, image b) distributed throughout the capsule interior. The charged PMA_{SH} capsule wall (pH 7.2) provides both steric hindrances to DNA diffusion[13] and also an energetic barrier for permeation of a negatively charged oligonucleotide through a negatively charged wall.[14]

Being stabilized solely through disulfide linkages, these capsules are deconstructed upon exposure to a thiol-disulfide exchange reagent, rapidly releasing the oligonucleotides into bulk solution (see the Supporting Information). Although in the current form these capsules exhibit burst-release characteristics, it should be possible to engineer the capsule properties to control the release of the cargo through rational design of the oligonucleotide length, capsule-wall thickness, and cross-linking density. We are currently investigating this to obtain release profiles to suit specific requirements.

In summary, we have developed a method to obtain monodisperse, degradable polyelectrolyte capsules filled with oligonucleotide sequences. The method permits essentially quantitative incorporation of the oligonucleotide into the capsules, which are stable for at least 72 h, and should allow facile control over capsule loading through variation of DNA to particle ratio. We also used this approach to encapsulate

plasmid DNA by using 3-μm silica template particles with either PMA/PVPON or DNA oligonucleotides[15] as the capsule/membrane components. The plasmid DNA released from these capsules behaves like native DNA in hybridization assays and enzymatic reactions. Details of these studies will be presented in a future publication.

Experimental Section

Synthesis of the oligonucleotide-filled capsules. A suspension of the SiO_2^+ particles (0.25 wt%) was combined with a TAMRA-$T_{15}C_{15}$ solution and allowed to interact for 15 min, after which time the suspension was charged with PMA_{SH} to a final concentration of PMA_{SH} of 0.5 mg mL^{-1}. After an incubation time of 15 min, the particles were separated through centrifugation and washed three times with acetate buffer solution (10 mM; pH 4). The particles were resuspended in 250 μL of pH 4 buffer solution through vortexing (no sonication was used at any step of assembly) and combined with 250 μL of the adsorbing polymer solution. The solutions of PMA_{SH} used in the adsorption cycles (acetate buffer solution (1 mg mL^{-1} in 10 mM; pH 4)) were prepared from the 10 mg mL^{-1} stock solution of PMA_{SH} incubated with dithiothreitol (DTT; 100 mg mL^{-1}) in phosphate buffer solution (10 mM; pH 8) for at least 12 h. After completion of the multilayer build up, the particles were exposed to a 2 mM solution of chloramines T in 2-(*N*-morpholine)ethanesulfonic acid (MES) buffer solution (pH 6) for 1 min, followed by two washing cycles with MES buffer solution (pH 6).To form hollow capsules, the silica core was dissolved by treatment with HF/NH_4F solution (2:8 M; pH 5) at 20 °C for 5 min, followed by multiple centrifugation (4500 *g* for 10 min)/buffer solution washing cycles.[12] The washing cycles were repeated as necessary until the pH of the capsule suspension became identical to the pH of the washing buffer solution.

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[10] An increase in TAMRA fluorescence was also observed while depositing PMA_{SH}/PVPON multilayers on SiO_2^+ particles with TAMRA-poly$T_{15}C_{15}$ by using PMA_{SH} without the AF488 dye. However, the magnitude of the fluorescence increase was much lower ($\approx$3 times) than that observed when the dye was present. Also, TAMRA fluorescence for the capsules remained greater than that for the SiO_2^+ particles with adsorbed oligonucleotide. These results show that the change in the local environment of TAMRA also plays an important role in the fluorescence observed.

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