Virus-Decorated Biocomposites

Engineering Virus Functionalities on Colloidal Polyelectrolyte Lipid Composites**

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The fabrication of functional supramolecular assemblies of nanometer dimensions consisting of both biological and artificial constituents is an exciting and rapidly developing new field at the intersection of biology, chemistry, and physics.

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From the standpoint of pure research it is challenging to study the structure and function of these "bionanocomposites". In addition, smart biocomposite systems for drug delivery and artificial vectors for gene therapy should have exciting applications in medicine.

In the course of evolution nature designed viruses, small particles that defy categorization as actual living entities or just small assemblies of biomolecules. Viruses actually are nanocomposites consisting of only a few polymeric species and often equipped with a lipid membrane. They carry genetic information for their replication but need a host cell to accomplish reproduction. The virus envelope, composed of less than a handful of macromolecular species, nevertheless, bears all the functions needed to recognize and enter a host cell. This is exploited in various biotechnological applications such as protein production, vaccine design, development of genetic libraries, and gene therapy.

If it would be possible to engineer colloidal particles with surfaces bearing the functionality of viruses, this would be a novel means for the delivery of a variety of materials into cells and tissues. A possible strategy would be to fabricate composite particles or capsules equipped on their surface with all the necessary virus functions for cell-membrane passage. This approach could be used, for example, to deliver a cocktail of material packed into a small colloidal entity into cells; this is not easy to do with the existing delivery devices. In this communication we describe how such virus-modified particles have been fabricated by the Layer-by-Layer (LbL) approach originally introduced for macroscopic surfaces,[1] and how their functionality has been proved.

Through the consecutive adsorption of oppositely charged polyions on colloidal particles, multilayers can be fabricated.[2] Their thickness and composition can be tuned on the nanometer scale. When this synthetic method is applied to colloids with soluble cores, capsules can be fabricated.[3] Various functions can be added by either employing functional species for adsorption or by subsequent modification.[4] Capsules can be loaded with different materials,[5] and lipid bilayers can be added.[6]

At the same time molecular biology, in particular, virology, provides a variety of tools for genetic engineering. Techniques for the manipulation of viruses are well established. Various proteins and peptides can be expressed at the virus surface.[7] These modified viruses thus provide great flexibility in the choice of biologically engineered surfaces. Combining the assembly of virus-like LbL colloids and capsules with genetic engineering thus would open novel pathways for fabricating functional bionanocomposites of complex but fully controlled interfacial composition. They may be useful as combinatorial entities in a variety of biomedical and biotechnological applications such as diagnostics, vaccination, and delivery.

In this work, Rubella-Like Particles (RLPs)[8] were employed as the virus-like material. These particles are identical copies of the lipid-enveloped rubella virus (RV) except without the virus RNA. The RLPs can be harvested from CHO cells transfected with an expression plasmid containing a cDNA of the RV subgenomic RNA. RV binds to its host cell surface, and induces endocytosis and fusion with

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the late endosomal membrane. Binding and fusion are mediated by the rubella virus membrane protein E1 present in a complex with E2, the only other membrane protein of the rubella virus. The E1 protein function is triggered by pH, a feature displayed by many lipid-enveloped viruses. Cell-membrane binding occurs at physiological pH, while fusion with the late endosomal membrane takes place under the acidic conditions inside the endosome. The fusion competence of the E1 protein[9] is activated at low pH.

The protocol for the fabrication of RLP-decorated LbL colloids is illustrated in Figure 1. Initially a polyelectrolyte multilayer consisting of poly(allylamine hydrochloride) (PAH) and poly(sodium 4-styrenesulfonate) (PSS) was assembled onto a colloidal core. A variety of cores may be used, which can be removed afterwards, resulting in hollow polymeric capsules. The next step consisted in adding a phosphatidylserine (PS) bilayer onto this polyelectrolyte multilayer cushion. Small unilamellar PS vesicles[10] were incubated with the LbL-coated colloids having as the top layer the positively charged PAH. A bilayer was formed spontaneously by vesicle adsorption and spreading. The existence and stability of this PS bilayer was proved by means of confocal laser scanning microscopy (CLSM).[11]

On incubation of PS-coated LbL colloids with RLPs at low pH (pH 4), the particles attached to the lipid layer by electrostatic forces and subsequently fused with the membrane, presenting RV envelope proteins E1 and E2 on the surface. Tryptophane fluorescence spectroscopy was used to detect the presence of the RLPs. When the RLP-coated colloids were transferred into a buffer with pH 7.4, those RLPs which did not fuse at pH 4 desorbed, as revealed by the tryptophane fluorescence in the supernatant (see Figure 2). The amino termini of the RV E1 and E2 proteins as well as the amino groups of the virus lipids, mostly sphingomyelin, were labeled with tetramethylrhodamine. Their localization was then studied by means of CLSM (see Figure 3). The presence of the virus components was demonstrated by the homogeneous fluorescent coat on the colloids. Furthermore, SDS polyacrylamide gel electrophoresis revealed the presence of the complete set of structural RV proteins on the colloids (data not shown).

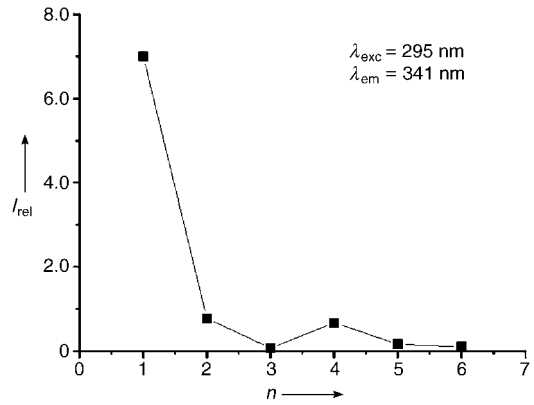

Figure 2. Tryptophane fluorescence intensity (I_{rel} in arbitrary units) in supernatants of washing cycles (n) following incubation of lipid polyelectrolyte composite particles with RLPs. Washings 1–3 were performed in 0.2 M phosphate/0.1 M citrate buffer at pH 4, washings 4–6 in 0.2 M phosphate/0.1 M citrate buffer at pH 7.4. RLPs that did not fuse with the lipid layer were desorbed and were detected by the small jump of fluorescence in the supernatant from washing cycle 4.

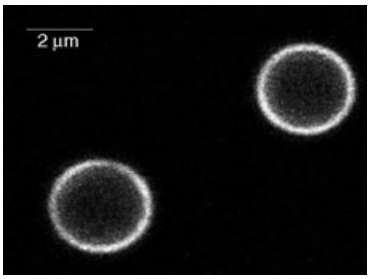

Figure 3. Fluorescence of rhodamine-labeled RLPs engineered on PS polyelectrolyte-coated silica particles.

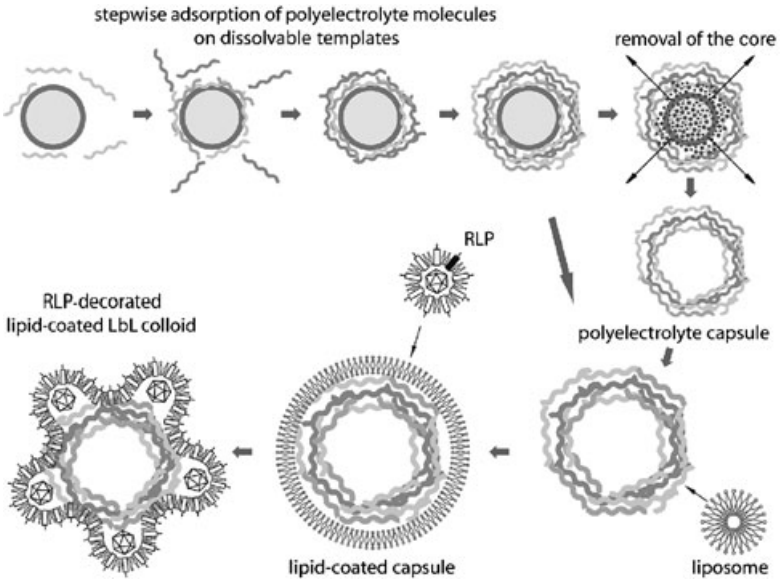

Figure 1. Protocol for engineering virus functionality on polyelectrolyte lipid composite colloids and capsules. Removal of the core is optional.

The octadecylrhodamine (R18) dequenching assay[12] was used to demonstrate the mixing of the virus membrane with the PS layer present on the colloids. The probe was added to RLPs in such a concentration that its fluorescence was partly quenched. Upon fusion it should mix with the bilayer of the target, resulting in dilution of the probe and partial dequenching of fluorescence. At pH 4 it was found that at least one-fourth of the applied RLPs had fused with the PS layer, whereas at neutral pH the observed small dequenching indicated fusion of not more than a few percent. In addition, Foerster energy transfer was demonstrated between the top polyelectrolyte layer containing FITC-labeled PAH and the R18 probe, which proves that the distance between the two labels is on the order of only a few nanometers. Because of the size of the capsid structure of the RLPs, significant energy transfer is possible only if the R18 probe

present in the RLP envelope is able to dilute laterally in the phosphatidylserine bilayer of the capsules.

Figure 4 reveals that E1 epitopes are indeed presented on the surface of the fabricated composite colloids. Here a monoclonal antibody raised against the major immunodominant epitope of E1 was applied. To reduce unspecific binding of the primary as well as secondary antibody to the particle surface, several blocking reagents were tested. Satisfying results were obtained with protamine sulphate, while the use of bovine serum albumine lead to considerable background signals.[13]

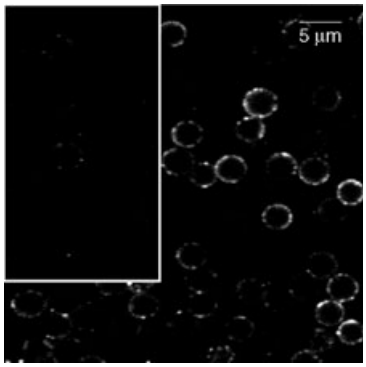

Figure 4. Confocal image of immunofluorescence against rubella virus E1 epitope on engineered silica particles. The presence of E1 is revealed by the fluorescence of the secondary antibody bound to the primary antibody against E1. The inset shows the control under identical fluorescence conditions.

The key function of the virus surface is the binding to a host cell surface, induction of endocytosis, and subsequent fusion with the late endosome membrane. In order to demonstrate the retained biological activity and functionality of E1, these engineered constructs were presented to living cells. As shown in Figure 5, LbL-coated colloids and capsules were taken up by endocytosis into Vero cells.[14] The endocytosed particles form clusters of fluorescence near the nucleus. The degree of uptake depends strongly on the nature of the top layer. While coated particles with PS as the top layer were rarely found inside the cells, those colloids which had been fused with the RLPs were effectively incorporated. The difference in uptake between the RLP-decorated PS and only PS as the top layer of the colloids can be clearly attributed to the presence of viral envelope proteins.

It is worth mentioning that particles with the polycation PAH on their surface were also found in large amounts inside the Vero cells. In this case, cell damage was observed. The uptake of particles with PAH as the top layer is probably initiated by their strong binding. This nonspecific binding was avoided when various negatively charged biopolyelectrolytes were utilized as the top layer. It is not known yet if they got stuck in the endosome or had gained access to the cytosol. This remains an exciting unresolved question for further experiments involving selective targeting and delivery of incorporated substances such as DNA[15] and proteins. Defoliation of the multilayer and improved biodegradability of the composite constituents are other issues for future research.

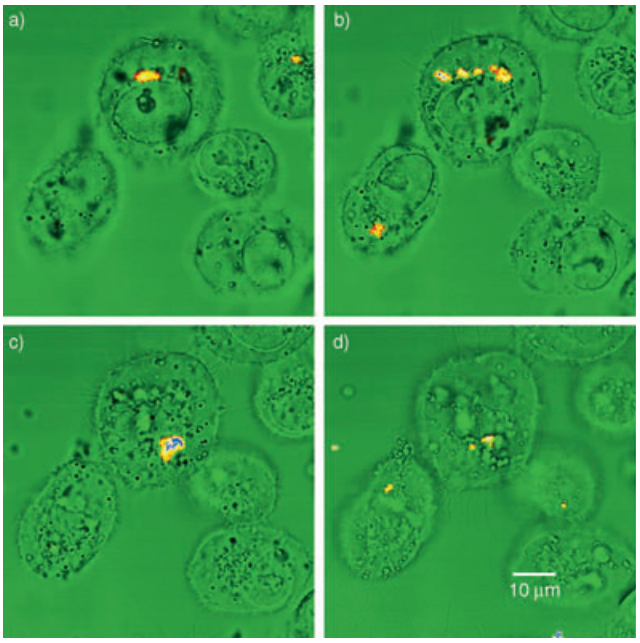

Figure 5. Uptake of 1-µm RLP-coated fluorescent LbL colloids (the fifth layer contained FITC-labeled PAH) into Vero cells. The images in a)–d) represent four consecutive confocal scans from top to bottom through the cultured Vero cells. Fluorescent colloids forming clusters near the nucleus can be identified by their red to bright yellow color. The blue color in the fluorescent spots indicates the overflow of intensity.

We conclude that the surface of lipid and polyelectrolyte multilayer coated colloids and capsules can be modified with rubella-like particles. It is very likely that this can be also achieved with other viruses or virus-like particles that infect cells by endosomal uptake.[16] This could be utilized potentially for the development of particle or capsule systems with targeting properties utilizing the specific binding properties of certain viral proteins. Moreover, with the well-developed techniques in genetic engineering, it should be possible to add membrane proteins of nonviral origin to the surface of viruses or virus-like particles. Hence, it may be possible to fabricate particles with virus functions at their surfaces along with other desired biological properties in designed arrangements on the nanometer scale. Considering all these features together, this technique could be a general approach for the transfer of biological functionalities of various kinds onto colloids, capsules, and flat surfaces.

Experimental Section

Materials: Silica particles with diameters of 3 µm and 1 µm were purchased from microparticles GmbH (Berlin, Germany). Poly(allylamine hydrochloride) ($M_w \approx 70000$) and poly(sodium 4-styrenesulfonate) ($M_w \approx 70000$) were purchased from Aldrich. L-α-Phosphatidylserine (brain, porcine; sodium salt; 20 mg mL^{-1} in chloroform) (PS) was purchased from Avanti Polar Lipids, Inc.

RLP preparation: RLPs were isolated according to the method of Hobman et al.[8] Briefly, permanent transformed CHO cells were grown on triple flasks (Nunc) at 37 °C in 5% CO_2 atmosphere. The RLPs were secreted into the supernatant of transfected cells. The

culture medium was concentrated initially using an Amicon hollow fiber equipment (H1-100) and subsequently with an Amicon stirring cell. The concentrated solution was processed on a Sepharose 4B-Cl chromatography column. Protein content was determined using the bichinonic acid assay from Sigma.

Addition of the lipid layer: The lipid bilayer was formed by adsorption of unilamellar vesicles to multilayer-coated colloids or capsules with PAH as the top layer. Vesicles and the substrates were stirred for 30 min at 37 °C on an Eppendorf Thermomixer Comfort in 0.1 M NaCl. After incubation, tween 80 (Serva, Heidelberg/New York) was added to a final concentration of 0.05 % (v/v), and the vesicles were incubated for another 5 min and subsequently washed four times with 0.1 M NaCl or PBS.

Fusion procedure: Fusion of rubella-like particles onto the lipid-coated colloids was conducted in 0.2 M phosphate/0.1 M citrate buffer at pH 4 by incubation with RLPs for 30 min at 37 °C followed by four washings with a 0.2 M phosphate/0.1 M citrate buffer, pH 7.4. For a total particle surface of 0.01 m^2, 200 μL RLPs in 0.2 M phosphate/0.1 M citrate buffer pH 4 (1 mg mL^{-1} protein) were used.

Octadecylrhodamine dequenching assay: The phospholipid content of the RLPs was estimated according to the method of Bardeletti et al.[17] The protocol for labelling RLPs with R18 (octadecyl rhodamine B, chloride salt; Molecular Probes, Inc.) was as follows: A 2-mM solution of R18 in methanol was added to RLPs in PBS corresponding to a final concentration of 4 % (mol/mol) R18 per lipid. The labeling reaction was performed at room temperature for 1 h in the dark. Subsequently, the RLPs were purified over a Sephadex G75 column. Fractions were analyzed by fluorescence spectroscopy for simultaneous occurrence of label and protein. Triton-X-100 (Merck, Darmstadt/Germany) was used at a final concentration of 0.1 % (v/v).

Immunofluorescence: Coating the particles with protamine sulfate (from herring, Sigma) prior to fixation with paraformaldehyde prevented unspecific binding of the primary antibody. Lipid polyelectrolyte particles as the negative control and particles coated with RLPs were incubated with 1 mL protamine sulfate (1 mg mL^{-1} in 0.1 M NaCl) for 10 min at room temperature. Three washings in PBS followed. The samples were spotted on a glass slide and incubated for 30 min with paraformaldehyde (2 % w/v). Subsequently, the primary antibody (Mab < Rubella > M-1B9-IgG, kindly provided by Roche Diagnostics, Germany) diluted 1:200 in PBS/1 % BSA, centrifuged for 10 min/14 krpm) was added, and the particles were incubated for another 2 h. The samples were washed five times in PBS for 10 min. Then the secondary antibody (Anti-mouse-IgG Cy3, Sigma #C 2181) diluted 1:75 in 1 % BSA in PBS, centrifuged at 10 min/14 krpm) was added. An incubation for 1 h and three washings in PBS each for 5 min followed.

Uptake in Vero cells: Vero cells were grown in Dulbecco's modified eagle medium alpha with Glutamax (Gibco BRL) on LAB-Tek slides (Nunc) to confluency. Coated particles 1 μm in diameter were pipetted to the cells in a ratio of 3:1, and the cells were incubated overnight at 37 °C in 5 % CO_2 atmosphere. Prior to examination the samples were washed three times with PBS.

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