COMMUNICATION
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Engineered protein cages for selective heparin encapsulation
A heparin-specific binding peptide was conjugated to a cowpea chlorotic mottle virus (CCMV) capsid protein, which was subsequently allowed to encapsulate heparin and form capsid-like protein cages. The encapsulation is specific and the capsid-heparin assemblies display negligible hemolytic activity, indicating proper blood compatibility and promising possibilities for heparin antidote applications.

Heparin (Fig. 1a) is a highly anionic polysaccharide belonging to the family of glycosaminoglycans and is widely used as an anticoagulant in surgical practices and thrombotic events. The anticoagulant activity is based on its ability to bind to and subsequently activate antithrombin-III, which leads to the inactivation of vital coagulation cascade substances such as thrombin and factor Xa. For safe clinical operation, heparin requires an antidote, which can counteract the anticoagulant effect and suppress potential side effects, if needed. Protamine sulfate is a small arginine-rich cationic protein that binds heparin via electrostatic interactions, and is commonly used for this purpose. This interaction is electrostatic in nature, but nonspecific, and protamine sulfate can cause multiple adverse effects. This has initiated diverse attempts to develop safer heparin antidotes, such as cationic polymers and small molecules, which all largely rely on the electrostatic binding with heparin.

Although efficient binding can be achieved, cationic platforms often lack specificity to heparin and usually exhibit high cytotoxicity. Furthermore, the complexes formed with heparin are often large and ill-defined aggregates. Therefore, more sophisticated alternatives that would transform heparin into small and well-defined particles, which are biologically inert, are needed.

Virus-like particles (VLPs) are protein assemblies derived from wild-type viruses. They lack the viral genome but have similar well-defined capsid structures as native virus particles, and have therefore been explored for biomedical and nanotechnological applications. Indeed, their most appealing features are the accurate sizes and shapes, which are combined with a relatively high stability and versatile modification potential. The removal of the viral genome also generates a highly confined cavity, which can be harnessed to compartmentalize other nanoscale substances. Cowpea chlorotic mottle virus (CCMV) capsid is one of the most studied VLPs as they can be produced in high yields and undergo pH and ionic strength-dependent swelling and assembly, which have therefore been intensively utilized in cargo-loading.

In more detail, CCMV is a plant-infecting icosahedral virus consisting of 180 capsid proteins (CPs) in its native $T=3$ form, leading to a capsid with an outer diameter of 28 nm and an inner cavity diameter of 18 nm (Fig. 1b). Its $T=1$ and pseudo $T=2$ forms consist of 60 and 120 capsid proteins and have diameters of roughly 18 and 22 nm, respectively. Each CP is formed from 190 amino acids with its N-terminus located on the interior of the capsid. CCMV can be modified in multiple different ways, for example, by functionalizing carboxylic acids or amines on the exterior of the capsid. To obtain a highly defined modification of the capsid interior, an appealing approach is to engineer the N-termini, which provides site- and number-specificity. For this purpose, an enzymatic method involving a reaction catalyzed by the enzyme Sortase A (SrtA) has been developed. This benign methodology was first utilized to label the CCMV capsids with a fluorescent tag, but...
due to the generic nature of the strategy, a wide range of cargos loaded in VLPs can be envisioned.33–35

So far, only the exteriors of virions have been employed to display polycationic motifs (poly-Arg) for heparin-binding.36–38 However, multiple mutations may yield unstable virions and lead to nonspecific electrostatic interactions with heparin. In this work, we report on the conjugation of a heparin-binding peptide (HBP), i.e. a heparin-specific sequence derived from the fibroblast growth factor having a dissociation constant of ~134 pM with heparin, to the interior of the CCMV capsid.39

The binding and packing of heparin into the protein cage was studied with dynamic light scattering (DLS), transmission electron microscopy (TEM), and fast protein liquid chromatography (FPLC). Assemblies were not observed in the presence of two other glycosaminoglycan analogues, demonstrating high selectivity towards heparin. Moreover, no hemolytic activity was detected, implying proper biocompatibility.

The particular HBP was selected based on its high selectivity and affinity to heparin, thus minimizing off-target binding even in complex biological environments.12,39 Conjugation of HBP to the CP N-terminus was realized with the SrtA-based method, as illustrated in Fig. 1c. SrtA catalyzes the cleavage of the HBP C-terminal sorting signal (LPETG) after threonine and the subsequent ligation with an N-terminal glycine in the CP. The detailed coupling procedure and characterization of the intermediate conjugates can be found in the ESI† (Fig. S1–S6, ESI†).

The final product was characterized by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry. Based on the SDS-PAGE and MALDI-TOF analysis, approximately 25% of the CPs were successfully conjugated with HBP (Fig. 1d and Fig. S7, ESI†), which corresponds on average to 45 modified CPs per one T = 3 CCMV capsid. Initially, TEM imaging was employed to investigate the morphology of the assemblies formed by HBP modified CPs alone. Negatively stained spherical VLPs were observed under the TEM microscope, having a diameter of ~28 nm, which corresponds well with the expected dimension of the T = 3 icosahedral capsids (Fig. 1e and Fig. S9a, ESI†). The presence of dark regions in the capsid cores indicates that the capsids are empty as the stain is able to enter the voids.40 Empty CCMV capsids were then disassembled by dialysis against Tris–HCl buffer (50 mM, pH = 7.4). Disassembly was confirmed by TEM and DLS measurements, which showed a size shift from 28 nm to 8–9 nm (Fig. 1f and Fig. S9b, ESI†). The obtained CP solution was then mixed with heparin in different quantities yielding heparin-loaded capsids (Fig. 1g).

As observed in Fig. 2a–c and Fig. S12, ESI† the heparin-loaded capsid assemblies formed with three different heparin to protein mass ratios (ρ = 0.02, 0.22, and 0.44) all had a roughly spherical morphology and a smaller diameter than the empty T = 3 capsid (28 nm). For instance, TEM images indicated an average size of 18.6 nm at ρ = 0.02 (Fig. 2d). Similar diameters were observed with the ρ = 0.22 (18.6 nm) and 0.44 (20.2 nm) samples (Fig. 2d), matching well with the dimension of T = 1 capsids. The preferential formation of T = 1 capsids has been observed previously, i.e. when highly negatively charged synthetic polymers40–43 and nucleic acids were encapsulated in CCMV VLPs.44,45 The particle size has been shown to depend on the molecular weight of the encapsulated compound, and commonly higher molecular weight compounds promote the formation of larger capsids.40,45 Unlike empty capsids, the heparin-loaded assemblies did not show dark cores (Fig. 2e), demonstrating that the capsid voids were filled and successfully loaded with heparin molecules. Cryo-TEM images of the heparin-loaded capsids further verified the spherical morphology and ~18 nm size of the complexes and confirmed that the observed structures are not a result of a drying effect.

Fig. 1  (a) Structure of the major repeating unit of heparin. (b) Drawing of the CCMV capsid (T = 3) structure. (c) Illustration of the Sortase A mediated coupling of HBP to CP. (d) MALDI-TOF spectrum and SDS-PAGE (inset) of HBP modified CPs. (e) TEM image of empty capsids formed by HBP-modified CPs. (f) DLS measurements (volume-weighted size distributions) of the empty capsid and free CP. (g) Schematic representation of heparin (yellow chain) encapsulation by a mixture of CP and HBP modified CP to form T = 1 structures.
The effect of the amount of heparin on capsid formation was quantified with fluorescein (FITC) moieties according to a previously published method. Successful heparin-FITC conjugation was confirmed with FPLC and nuclear magnetic resonance (NMR) spectroscopy (Fig. S8, ESI†). Fig. 2g shows that the free CPs were eluting at 19.6 mL and 17.1 mL. Comparison of the peak areas revealed that 71% of protein was eluting at the higher elution volume (19.6 mL, Fig. S11, ESI†), which corresponds to the protein dimers. These values are well in line with previously published work conducted under similar conditions where the CP dimers were eluting at 18.5 mL. In the present studies, another peak at 17.1 mL, corresponding to slightly larger capsomers, was also observed. The assembled capsids were expected to elute at lower volumes, and for the ρ = 0.02 sample, a minor broad peak appeared around 13.9 mL (Fig. 2g), which corresponds to T = 1 capsids. However, further addition of heparin (ρ = 0.44) did not significantly increase the fraction of T = 1 capsids. Instead it enhanced the amount of the intermediate particles as observed by the larger peak area between 17.1 mL and 13.9 mL. Additionally, unbound heparin (peak at 22.4 mL) started to appear (Fig. S12d, ESI†), indicating that the excess of heparin did not induce further formation of capsids. This finding is supported by previous studies, which have shown that capsid formation depends on the amount of the encapsulated compound and that high cargo ratios limit capsid formation. Fractions corresponding to the T = 1 capsids (13 mL) were collected and investigated with the help of fluorescence spectroscopy measurements. A clear emission spectrum from the fluorescein-conjugated heparin was observed for all the heparin-loaded samples, while that for pure heparin-FITC was negligible, demonstrating that the encapsulations had been successful (Fig. 2g inset and Fig. S13, ESI†).

The selectivity of the heparin encapsulation was evaluated qualitatively with DLS. Studies aimed at encapsulating other negatively charged target molecules were conducted with two glycosaminoglycan analogues, i.e., hyaluronic acid and chondroitin sulfate as well as bovine serum albumin. No size change was observed when these compounds were added to CCMV CPs (ρ = 0.02–0.22), indicating that capsids or other higher-order structures are not formed (Fig. 3a). However, adding heparin in similar mass ratios clearly induced T = 1 capsid formation, which is visible as a peak between the CP dimers and T = 3 capsid (Fig. 3b and Fig. S14, ESI†). Adding heparin to the preassembled T = 3 capsid did not result in any changes in the size distribution indicating that heparin is not bound to the exterior of the capsid. Such observation is expected since the empty capsid remains assembled only at pH 5 in the presence of high electrolyte concentration (500 mM NaCl and 10 mM MgCl₂), which efficiently screens electrostatic interactions.

Furthermore, we found negligible hemolytic activity of the CPs up to 0.5 mg mL⁻¹ concentration on red blood cells (RBCs) (Fig. 3c), which is in sharp contrast to the effect of commonly employed protamine sulfate, thus providing a promising strategy in heparin neutralization.

In conclusion, we have shown that CCMV capsid proteins modified with a heparin-binding peptide exhibit specific heparin
binding at physiological pH's. Co-assembly with heparin yielded $T = 1$ capsids with diameters ranging between 18-20 nm according to the DLS measurements and TEM characterization. FPLC results further confirmed the capsid formation and revealed a heparin concentration-limiting effect on capsid formation. The binding of heparin was proved to be specific as no capsid formation was induced with two other glycosaminoglycan analogs. Moreover, hemolysis assays demonstrated a better biocompatibility than the commercial heparin antidote protamine sulfate. These results indicate that VLPs are promising materials for heparin binding, especially when the binding is further optimized for physiological conditions and after thorough hematologic studies have been conducted.

Conflicts of interest
The authors declare no conflicts of interest.

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