

A VIRUS-BASED SINGLE-ENZYME NANOREACTOR

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Introduction

Significant developments in biology and biochemistry made viruses readily available and easy to handle. Recently, virus-based nano-particles have been used as interesting tools in the construction of materials.¹ In our ongoing investigations, we intend to use the viral capsid of the cowpea chlorotic mottle virus (CCMV) as a building block in the construction of nanometer sized architectures that can function as reactors or display special material properties.²

Chemical transformations in cells take place with remarkable precision in both space and time. Almost all these processes are catalyzed by enzymes, and as a consequence their study is of great importance for the detailed understanding of reactions in life. The investigation of single molecule processes has significantly contributed to this field, however, the vast majority of these studies were carried out under highly artificial conditions, e.g., extreme dilution and on enzymes chemically or physically anchored to a surface.³ On this paper we report the investigation of single enzyme processes on enzymes which are contained by a protein cage of a plant virus, consequently obtaining a more natural environment. In order to carry out these studies, the enzyme horseradish peroxidase (HRP) is, for the first time, incorporated into the inner cavity of the CCMV capsid. Subsequently, the enzymatic activity of the HRP molecules inside the CCMV capsid is examined at the single molecule level in order to investigate the influence of the container on the HRP activity.

Results and Discussion

CCMV virions have an outer diameter of 28 nm; the protein shell defines a precise inner cavity with a diameter of ca. 18 nm. The virus is composed of 180 identical coat protein subunits which encapsulate the viral RNA.⁴ An interesting feature of the CCMV virus is its sensitivity to pH and ionic strength.⁵ CCMV virions can, depending on pH, rapidly disassemble *in vitro* into protein dimers and RNA. After removal of RNA and with a change in pH, the purified viral coat protein subunits will easily self-organize and reform the capsids (Fig. 1a).^{6,7} The reversible pH dependent assembly of the CCMV capsid provides a unique molecular gating mechanism to control the containment and release of entrapped material. We have utilized such a mechanism to encapsulate the enzyme HRP. These processes were monitored by transmission electron microscopy (TEM) and fast performance liquid chromatography (FPLC). Initially, the capsid was transformed into capsid protein dimers by dialyzing the solution against Tris buffer (pH 7.5), indicated by a signal at $V = 1.78$ mL in the FPLC (Fig. 1b). After dialysis, the enzyme was added, in excess, to the capsid protein dimer solution. Subsequently, the mixture was dialyzed against acetate buffer (pH 5) in order to re-assemble the virus capsid while incorporating the guest enzyme.

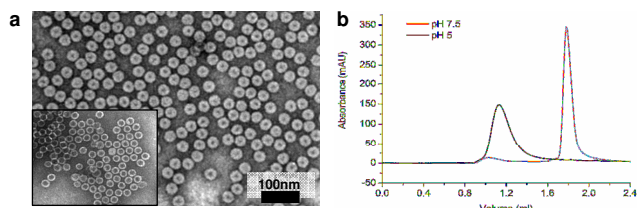


Figure 1. Characterization of CCMV and CCMV capsid. **a**, TEM (negative staining) of the CCMV virus and the empty capsid (inset). **b**, Size-exclusion FPLC of the CCMV protein at pH 5 (black curve) and at pH 7.5 (red curve).

FPLC was used to purify the HRP-containing capsid (elution volume $V = 1.12$ mL) from the excess of HRP (elution volume $V = 1.8$ mL) (Fig. 2a). Co-elution of the guest enzyme and capsid was observed when concentrated fractions from the FPLC were analyzed by fluorescence spectroscopy. The HRP utilized in the experiments was labeled with Alexa Fluor 532 dye ($\lambda_{exc} = 530$ nm). The CCMV capsid containing this guest enzyme displayed significant emission when excited at $\lambda = 530$ nm (Fig. 2b), indicating that inclusion of the enzyme had occurred. Test experiments using a colorimetric assay (2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and H_2O_2) revealed that the HRP-containing CCMV capsid still possessed substantial enzymatic activity, further proving the successful encapsulation of the enzyme.

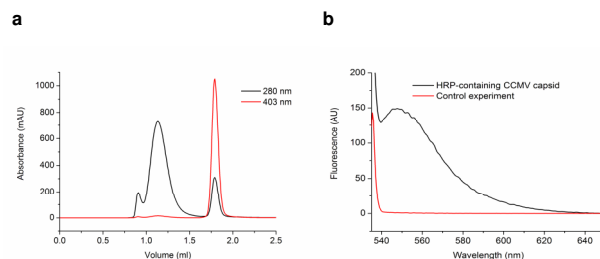


Figure 2. Inclusion of HRP in the virus capsid. **a**, Size-exclusion FPLC of the HRP-containing CCMV capsid. **b**, Emission spectra ($\lambda_{exc} = 530$ nm) of the HRP-containing CCMV capsid fraction and the corresponding fraction of the control experiment (the CCMV capsid devoid of HRP).

The enzymatic activity of the HRP molecules inside the CCMV capsid was examined at the single molecule level (Fig. 3).⁸ Two solutions were prepared: one containing HRP encapsulated inside the CCMV capsid and a control solution containing a mixture of empty CCMV capsid and free HRP. The solutions were deposited on separate hydrophilic cover slips, and, after addition of a mixture of hydrogen peroxide and the pre-fluorogenic substrate dihydrorhodamine 6G, were examined with a laser scanning confocal microscope. Dihydrorhodamine 6G acts as a hydrogen donor in the enzymatic reduction of hydrogen peroxide by HRP, yielding the highly fluorescent product rhodamine 6G. Images of the activity of HRP inside CCMV capsid after 10 minutes incubation of the mixture are shown in Fig. 3b. Bright diffraction-limited spots are clearly visible, which are attributed to diffusion of the substrate through the capsid pores, conversion and accumulation of product molecules in the capsid. During scanning of several images a decrease in fluorescence intensity due to photo bleaching and an increase of the background fluorescence is visible, which indicates the continuous formation of product molecules slowly leaking from the capsids into the solution. An image of the mixture of non-encapsulated HRP and empty CCMV capsid during catalysis is shown in Fig. 3d. Localized fluorescence is again observed, albeit at a much reduced intensity, however, no sign of accumulation of product molecules is detected in this case. Interestingly, in a blank experiment in which HRP was deposited in the absence of the CCMV capsid, no fluorescence was observed as a result of denaturation of the enzyme on the glass.

In order to further prove that the bright fluorescent spots in Fig. 3b are the result of enzymatic activity localized inside the capsid, the fluorescence of one of these bright spots was followed in time for both experiments. The autocorrelation curves were extracted from the time traces and while the HRP-included CCMV capsid complex fits well with a diffusion model, the same model cannot describe the autocorrelation curve for the non-encapsulated HRP enzyme. In this case, the curve is best fitted by a model for a chemical equilibrium.⁹ This, together with the observation that the activity is localized, led us to conclude that for the last case the HRP enzyme molecules are adsorbed to the outside of the capsid.

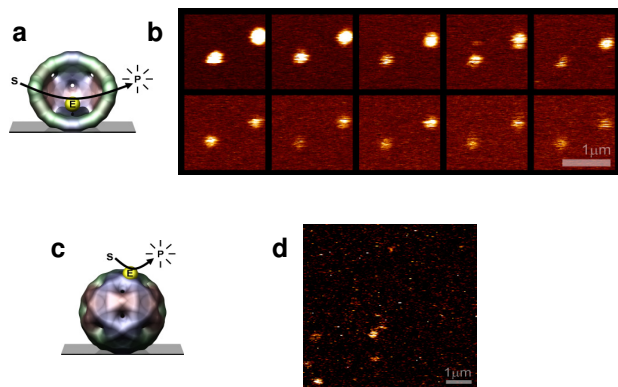


Figure 3. Single-capsid experiments. **a, c,** Schematic representation of the enzyme HRP inside the capsid (**a**) during enzymatic conversion of dihydrorhodamine 6G (s) to rhodamine 6G (p) and on the outside of the capsid (**c**). **b,** Consecutive scanning confocal fluorescence images of HRP encapsulated in CCMV capsid during the catalytic conversion of dihydrorhodamine 6G. The first image shows accumulated product molecules, which, in time, are bleached until a constant level is obtained. During this time the background fluorescence level increases. The time interval between images is 144 s. **d,** A mixture of non-encapsulated HRP and CCMV capsid shows localized activity; no product accumulation is observed.

Conclusions

Using the described disassembly/assembly cycle we have shown that an active enzyme can be loaded into the CCMV capsid by a statistical process in which the number of encased proteins is determined by the starting concentration of enzyme. Under the applied experimental conditions, i.e., concentration of the enzyme, it can be assumed that, as aimed, not more than one HRP enzyme molecule is encapsulated within an empty CCMV particle. This allowed the investigation of HRP kinetics on the single enzyme level, while the biocatalyst was encased in the virus capsid.¹⁰ The influence on the inclusion of guest molecules into the capsid under different conditions, e.g., concentration, protein size and surface charge, is currently under investigation. The ability of the capsids to encase enzymes and other materials, in combination with the possibility to introduce functionality on the protein surface, opens the way to systematically increase the complexity of the system while still allowing a detailed study of the single transformations under more realistic biomimetic conditions than previously reported by us and other groups.¹

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