Site-specific targeting of enterovirus capsid by functionalized monodisperse gold nanoclusters

Varpu Marjomäki\textsuperscript{a,b}, Tanja Lahtinen\textsuperscript{b,c}, Mari Martikainen\textsuperscript{a,b}, Jaakko Koivistob\textsuperscript{b,c}, Sami Malolab\textsuperscript{d}, Kirsi Salorinne\textsuperscript{b,c}, Mika Pettersson\textsuperscript{b,c}, and Hannu Häkkinen\textsuperscript{b,c,d,1}

Departments of \textsuperscript{a}Biology and Environmental Science, \textsuperscript{b}Chemistry, and \textsuperscript{c}Physics, and \textsuperscript{d}Nanoscience Center, University of Jyväskylä, FI-40014 Jyväskylä, Finland

Edited* by Roger D. Kornberg, Stanford University School of Medicine, Stanford, CA, and approved December 18, 2013 (received for review June 9, 2013)

Development of precise protocols for accurate site-specific conjugation of monodisperse inorganic nanoparticles to biological material is one of the challenges in contemporary bionanoscience and nanomedicine. We report here a successful site-specific covalent conjugation of functionalized atomically monodisperse gold clusters with 1.5-nm metal cores to viral surfaces. Water-soluble Au\textsubscript{102}(para-mercaptobenzoic acid)\textsubscript{44} clusters, functionalized by maleimide linkers to target cysteines of viral capsid proteins, were synthesized and conjugated to enteroviruses echovirus 1 and coxsackievirus B3. Quantitative analysis of transmission electron microscopy images and the known virus structures showed high affinity and mutual ordering of the bound gold clusters on the viral surface and a clear correlation between the clusters and the targeted cysteine sites close to the viral surface. Infectivity of the viruses was not compromised by loading of several tens of gold clusters per virus. These advances allow for future investigations of the structure–function relations of enteroviruses and enterovirus-related virus-like particles, including their entry mechanisms into cells and uncoating in cellular endosomes.

Results and Discussion

Our synthesis and targeting strategy combined two steps: (i) utilization of water-soluble monodisperse gold clusters, namely the structurally known (5) and theoretically well-understood (6) Au\textsubscript{102}(pMBA)\textsubscript{44} cluster (pMBA stands for \textit{para}-mercaptobenzoic acid) and (ii) the use of maleimide as the functional molecule on the gold marker to target the cysteine residues on the viral surface as the covalent binding site, schematically shown in Fig. 1. The water-soluble monodisperse Au\textsubscript{102}(pMBA)\textsubscript{44} clusters were synthesized by a method described by Kornberg and coworkers (7) using \textit{para}-mercaptobenzoic acid as the protective ligand layer. This one-step synthesis provided 2.8-nm-sized particles with 1.5-nm metal cores, which were characterized and confirmed to be monodisperse by TEM (\textit{SI Appendix}, Fig. S1), NMR (\textit{SI Appendix}, Fig. S2), and UV-visible (UV-vis) spectroscopy (\textit{SI Appendix}, Fig. S3) as well as gel electrophoresis (\textit{SI Appendix}, Fig. S4). The \textit{para}-mercaptobenzoic acid ligand of the Au\textsubscript{102}(pMBA)\textsubscript{44} cluster provides a convenient carboxylic acid functionality, which was used in ester bond condensation with a terminal alcohol. In our case, a six-carbon alcohol with terminal maleimide functionality provided the needed length to function as the linker molecule. Therefore, a modified Stieglch esterification reaction of N-(6-hydroxyhexyl)maleimide (NMR spectrum in \textit{SI Appendix}, Fig. S5) with Au\textsubscript{102}(pMBA)\textsubscript{44} cluster was used to synthesize the water-soluble maleimide functionalized gold clusters of Au\textsubscript{102}(pMBA)\textsubscript{44}N-(\textit{N}-hexyl 4-mercaptobenzoate)maleimide)\textsubscript{⧧}, hereafter called Au\textsubscript{102}-MI. The success of the functionalization was confirmed by gel electrophoresis (\textit{SI Appendix}, Fig. S4), UV-vis (\textit{SI Appendix}, Fig. S3), and IR spectroscopy (\textit{SI Appendix}, Fig. S6). The amount of linker molecules...
The diameter of the Au102(pMBA)44 is 2.8 nm, and CVB3 to target the free thiols of the cysteines close to the viral surface. CVB3, the targeting is likely to be mediated by the natural dynamics of the viral surface, opening momentarily thiol accessibility and facilitating the Michael reaction. This kinetic effect may also explain the rather long incubation times (a few hours to 1 d; see SI Appendix, Fig. S8) needed for achieving samples where most viruses are loaded with gold clusters. These observations are in line with previous results on poliovirus by other groups that showed dynamic access of exogenously added antibodies against polypeptides located inside the virus capsid (10), connected to a “breathing” mechanism. This natural breathing dynamics of the viruses (10, 11) may play an important role also for the conjugation of the Au102-MI and is a topic of further investigations.

We also addressed the issue of whether binding of tens of Au102-MI clusters to the viral surface affected the infectivity of viruses. We studied infectivity by end-point titration in GMK cells. Quantitative data on infectivity were obtained from end-point titration resulting in the number of infective virus particles in the virus preparations. Comparison of the virus preparations with or without the functionalized gold clusters showed that 1–2 d of incubation of virus with gold clusters at 37 °C did not lower the infectivity in GMK cells (Fig. 4). To assess a typical labeling outcome, we counted, for example for CVB3, more than 400 clusters after 48 h of incubation (356 out of 430 virus particles). In 79.3% of viruses being decorated with functionalized gold clusters, infection of GMK cells was observed. A typical labeling of incubation of virus with gold clusters at 37 °C did not lower the infectivity of viruses. 

The spatial distribution and cysteine–cysteine distance distributions are different in EV1 and CVB3. The fact that the cluster–cluster distributions reflect well these differences (Fig. 3) is the most convincing evidence of cysteine-specific binding of the functionalized clusters to both virus types. It is also important to consider the open environments adjacent to the sulfur of the cysteines, which has been analyzed in SI Appendix, Figs. S15 and S16 (the details of this analysis method are discussed in SI Appendix). The analysis shows that the most accessible cysteines are cys73 of EV1 and cys234 of CVB3. Because binding of the clusters to other cysteines is observed as well for both EV1 and CVB3, the targeting is likely to be mediated by the natural dynamics of the viral surface, opening momentarily thiol accessibility and facilitating the Michael reaction. This kinetic effect may also explain the rather long incubation times (a few hours to 1 d; see SI Appendix, Fig. S8) needed for achieving samples where most viruses are loaded with gold clusters. These observations are in line with previous results on poliovirus by other groups that showed dynamic access of exogenously added antibodies against polypeptides located inside the virus capsid (10), connected to a “breathing” mechanism. This natural breathing dynamics of the viruses (10, 11) may play an important role also for the conjugation of the Au102-MI and is a topic of further investigations.

We also addressed the issue of whether binding of tens of Au102-MI clusters to the viral surface affected the infectivity of viruses. We studied infectivity by end-point titration in GMK cells. Quantitative data on infectivity were obtained from end-point titration resulting in the number of infective virus particles in the virus preparations. Comparison of the virus preparations with or without the functionalized gold clusters showed that 1–2 d of incubation of virus with gold clusters at 37 °C did not lower the infectivity in GMK cells (Fig. 4). To assess a typical labeling outcome, we counted, for example for CVB3, more than 400 virus particles from randomly picked TEM images. This resulted in 79.3% of viruses being decorated with functionalized gold clusters after 48 h of incubation (356 out of 430 virus particles). Therefore, even if there are roughly 20% of viruses that are not conjugated with gold clusters, these results imply that gold
cluster conjugation keeps the viruses more stable, closer to the values of fresh virus taken directly from the freezer.

Linking colloidal gold nanoparticles and phosphine-functionalized gold nanoclusters to biomolecules has been a topic of wide interest since the 1980s (12–14). More recently, gold–cysteine interactions have been used for rigid labeling of proteins and DNA (15–17) and as facilitators for gold self-assembly at viral scaffolds for sensing and nanoelectronics (18, 19). Our work

![TEM images of CVB3 viruses (A–C) treated with functionalized and nonfunctionalized gold clusters. (A) After incubation for 2 d with Au102-MI clusters without column purification, (B) after column purification, (C) control with conventional negative staining of virus sample incubated with nonfunctionalized Au102(pMBA)44, and (D) EV1 incubated with Au102-MI for 1 d. Negative staining has been used also in D, resulting in the dark gray halo around the cluster. TEM magnification: (A) 2 × 10^5, (B) 2 × 10^5, (C) 1.5 × 10^5, and (D) 1 × 10^5. Note that the Au102-MI conjugated viruses appear larger than the nonconjugated virus. This results from two factors. On one hand, the particles of PTA salt are able to enter the grooves of the virus capsid in C. On the other hand, only the linker arm of the maleimide group can penetrate into the virus capsid, but the metal nanocluster part will remain outside. This makes the cluster–virus conjugates appear larger in A, B, and D.

![Quantitative and positions analysis of the Au102-MI clusters in the TEM images of cluster–virus conjugates. (A) Cluster–cluster distances compared with the thiol–thiol distances of cysteine residues in the known structure of EV1. In the panels on the right, “Exp.” shows the experimental data for the distribution of the cluster centers (marked by red dots on the left) and “Model” shows the calculated distance distribution of the thiol binding sites in the cysteines close to the viral surface. (B) Identification of binding patterns of the gold clusters in EV1, compared with thiol positions denoted by yellow spheres. (C) The same as A but for CVB3. (D) The same as B but for CVB3. In all of the analysis, three cysteine shells closest and most accessible to the viral surface are used (cys256, cys209, and cys73 for EV1 and cys134, cys234, and cys73 for CVB3). More details of the analysis are given in SI Appendix. TEM magnification: (A and B) 2.5 × 10^5 and (C and D) 2 × 10^5.]

Marjomäki et al. PNAS | January 28, 2014 | vol. 111 | no. 4 | 1279
The stronger binding will allow also biodistribution studies where one needs to track viruses along the normal infective route, e.g., through intestine to target tissues.

There are very few data yet available to understand enterovirus uncoating and genome release in cellular structures. Gold nanoclusters will allow for a better visualization of viruses in complex endosomal structures, which may help in understanding the biochemical and structural factors leading to successful opening of the virus to release the genome. With simultaneous labeling of virus receptor (e.g., α2β1 integrin for EV1), one may evaluate the spatial location of the virus in relation to the receptor or the limiting membrane of the endosome. In addition, gold nanoclusters allow potentially detailed (i) spectroscopic measurements of nanoclusters moving in relation to each other upon adding biochemical factors that lead to virus opening or (ii) manipulation of virus structure and function by using the fact that the clusters are strong absorbers of near-infrared radiation and generators of “local heat” upon deexcitation. Future work to refine the synthesis of maleimide linkers with tunable-length arms and impose fewer linkers per gold cluster by using ligand-exchange kinetics (20, 21), combined with control of concentration of clusters and time of incubation with viruses, may yield improved control of targeting desired cysteine groups at the surface of enteroviruses, e.g., close to positions that are currently thought of as critical for virus uncoating based on in vitro studies (22–24).

## Materials and Methods

All materials were commercial unless otherwise mentioned and were used without further purification. N-(6-hydroxyhexyl)maleimide (25) and Au$_{102}$(pMBA)$_{44}$ (7) were prepared according to literature procedures. NMR spectra were recorded with Bruker Avance 500 MHz or Bruker Avance 400 MHz spectrometers at 298 K, and the chemical shifts were calibrated to the residual proton resonance of the deuterated solvent. Mass spectra were measured with a Micromass LCT ESI-TOF instrument. Gel electrophoresis visualization was run on an 18% polyacrylamide gel (19:1, acrylamide:bisacrylamide) using 1× TBE buffer in a Bio-Rad Mini-Protean Tetra System gel electrophoresis apparatus at 120 V.

### Synthesis of N-(6-hydroxyhexyl)maleimide.

A stirred solution of 6-amino-hexanol (380 mg, 3.24 mmol) in saturated aqueous sodium bicarbonate (17 mL) was cooled to 0 °C in an ice bath. N-(methoxycarbonyl)maleimide (500 mg, 3.22 mmol) was added in small portions, and the resulting solution was stirred at 0 °C for 30 min. The ice bath was then removed, and the solution was allowed to warm to room temperature over 20 min. The aqueous layer was extracted with chloroform (4 × 20 mL), and the combined organic layers were dried over MgSO$_4$. Rotary evaporation gave a white solid, which was purified by column chromatography on silica gel, eluting with dichloromethane-methanol (20:1) as a white solid. The NMR parameters were as follows: $^1$H NMR (CDCl$_3$, 500 MHz) δ 6.65 (s, 2H), 3.57 (t, J = 6.55 Hz), 3.47 (t, J = 7.23 Hz), 1.87 (br s, 1H), 1.58–1.48 (m, 4H), 1.34 (m, 2H), 1.26 (m, 2H) ppm. $^{13}$C NMR (CDCl$_3$, 126 MHz) δ 170.3, 134.0, 62.5, 37.6, 32.4, 28.3, 26.3, 25.1 ppm. ESI-MS [M+Na]$^+$: m/z 220.

### Synthesis of Au$_{102}$(pMBA)$_{44}$.

The thiolate monolayer protected monodisperse Au$_{102}$(pMBA)$_{44}$ clusters were prepared according to previously published synthesis procedure using p-mercaptobenzoic acid (pMBA) as the protecting ligand. The purity and monodispersity of the prepared Au$_{102}$(pMBA)$_{44}$ clusters were determined by $^1$H NMR (400 MHz, D$_2$O-D$_2$NaOH) and UV-vis spectroscopy, gel electrophoresis, and transmission electron microscopy.

### Synthesis of Au$_{102}$.MI.

A solution of N-(6-hydroxyhexyl)maleimide (0.74 mg, 3.73 μmol) in dry dichloromethane (DCM) (1 mL) was added to a presonicated Au$_{102}$(pMBA)$_{44}$ (2 mg, 0.0745 μmol) in dry DMSO (5 mL) in a 50-mL conical. The mixture was vigorously stirred for 20 min. A solution of 1,4,N$_2$V dicyclohexylcarbodiimide (0.77 mg, 3.73 μmol) in dry DCM (1 mL) was then added dropwise to the cooled mixture while stirring. The stirring was continued overnight, and then the mixture was centrifuged at 3500 rpm for 5 min (Heraeus Labofuge 400, Thermo Scientific). The supernatant was transferred to a new 50-mL conical, and the maleimide-functionalized gold clusters were precipitated from the solution by adding solid NH$_4$OAc (73 mg, 0.947 mmol) and methanol (20 mL). The contents were mixed by shaking the conical and

Figure 4. The amount of infective (Upper) EV1 and (Lower) CVB3 quantified by the endpoint dilution assay. EV1 or CVB3 was treated with Au102-MI gold clusters and control clusters for 24 or 48 h at 37 °C and serially diluted on GMK cells to evaluate the infectivity of the virus preparations. Control virus was either fresh virus directly from the stock or virus that was incubated at 37 °C without gold clusters. The results are calculated as 50% tissue culture infective dose (TCID50).

...
then centrifuged at 3500 rpm for 10 min. The precipitates were collected, dried in air, and dissolved in ultrapure water. The product and the success of the N-(6-hydroxyhexyl)maleimide functionalization of the Au102(pMBA)44 cluster was analyzed by gel electrophoresis visualization on an 18% polyacrylamide gel, UV-vis, and IR.

**Viruses.** EV1 (Faruok strain) and CVB3 (Nancy strain) were obtained from ATCC. The viruses were propagated in GMK cells and purified using sucrose gradient as described (26). The infectivity of the purified virus was determined by end-point titration. The purity and RNA and protein content were determined by spectrophotometric analysis and protein measurement using the Biorad method. Column purification of virus–gold clusters preparations was done using the Sephadex G-25 columns (NAP-5) according to the manufacturer’s protocol (GE Healthcare). Briefly, 150 μL of the virus–gold suspension was added on top of the column, which was previously balanced with salt solution (137 mM NaCl, pH 7.0). After administering 350 μL of the salt solution, 5 × 100 μL fractions were eluted by adding salt solution in 100-μL increments. A small amount of gold eluted from the column concomitantly with the virus, while the bulk of the unbound gold was left on top of the column and did not elute during extensive washing. Gold clusters were recorded from the eluates with absorbance at 405 nm (OD405), and the amount of infective virus particles in the eluates was measured by end-point titration.

**Virus–Gold Conjugation.** EV1 or CVB3 purified by sucrose density gradient fractionation was used for gold conjugation. Gold clusters (45 μL, either Au102–MI or control Au102) were added to the virus samples in equal amounts (based on OD405 values). Binding was performed in the presence of 137 mM NaCl for various time periods at 37 °C.

**Virus Infectivity Measurement by the 50% Tissue Culture Infective Dose.** The endpoint dilution assay quantifies the amount of virus required to kill 50% of the infected hosts, here GMK cells. First, GMK cells (5 × 10^5 cells/mL) are plated the previous day, and serial 10-fold dilutions of the virus samples are added. After 3 d of incubation, the percentage of cell death (i.e., infected cells) is manually observed after crystal violet staining (10-min staining with crystal violet containing 10% formaline, followed by a wash with water to detach dead cells), which reveals intact (noninfected) cells, and recorded for each virus dilution. The 50% tissue culture infective dose (TCID50) is calculated by comparing the number of infected and uninfected wells of four replicates of the same virus concentration. The concentration at which half the wells would statistically be infected is extrapolated (TCID50).

**TEM Analysis.** Virions were visualized by TEM using negative staining. First, 3 μL (containing about 1 × 10^10 virus particles) of the virus was bound on formvar-coated grids, which were glow-discharged using an EMS5/SC7620 Mini sputter coater. After a 15-s incubation, excess sample was blotted away by carefully touching the drop with a blotted paper (Whatman 3MM). Negative stain (5 μL of 1% PTA solution) was added on the grid, and after 1 min, excess dye was blotted away as before. After air drying, samples were visualized using a TEM JEOL JEM1400. The images were recorded by using a bottom-mounted Quemesa CCD camera with 4008 × 2664 pixel resolution. Particle centers in Fig. 3 were identified using ImageJ (27).

**Spectroscopy.** IR spectra were measured with a Nicolet Magna-IR 760 FTIR spectrometer using 2-cm⁻¹ resolution and averaging 200 scans. UV-vis spectra were measured with a Perkin-Elmer Lambda 850 spectrometer using 2-nm resolution.

**Acknowledgments.** We thank H. Ojaniemi for preliminary synthetic work, A. Honkimaa for preliminary conjugation experiments on EV1 with nonfunctionalized gold clusters, and S. Mustalahti for helping in TEM imaging. V.M. wishes to thank S. Hafenster and her group for helpful discussions on EV1 structure. This research is supported by the Academy of Finland (Grant 257125 to V.M. and Grant 128341 to H.H.) and the Finnish National Graduate School on Computational Chemistry and Spectroscopy (LASKEMO) (I.K.).
Supporting Information for

Site-Specific Targeting of Enterovirus Capsid by Structurally Precise Functionalized Gold Nanoclusters

Varpu Marjomäki\textsuperscript{1,2}, Tanja Lahtinen\textsuperscript{2,3}, Mari Martikainen\textsuperscript{1,2}, Jaakko Koivisto\textsuperscript{2,3}, Sami Malola\textsuperscript{2,4}, Kirsi Salorinne\textsuperscript{2,3}, Mika Pettersson\textsuperscript{2,3}, Hannu Häkkinen\textsuperscript{2,3,4*}

\textsuperscript{1}Department of Biology and Environmental Science, University of Jyväskylä, FI-40014 Jyväskylä, Finland.
\textsuperscript{2}Nanoscience Center, University of Jyväskylä, FI-40014 Jyväskylä, Finland.
\textsuperscript{3}Department of Chemistry, University of Jyväskylä, FI-40014 Jyväskylä, Finland.
\textsuperscript{4}Department of Physics, University of Jyväskylä, FI-40014 Jyväskylä, Finland.

*Corresponding author: Hannu Häkkinen, Departments of Physics and Chemistry, Nanoscience Center, University of Jyväskylä, FI-40014 Jyväskylä, Finland
phone: +358 400 247973, email: hannu.hakkinen@jyu.fi
Modeling of the solvent accessible volume around cysteine thiol sites and the binding site distances. We used full virus models, taken from references (8) and (9), in the solvent accessible volume calculations. Van der Waals radius representing the approximate interaction radius of atoms were taken by default from the VMD program (e.g., 1.9 Å for sulfur atoms). For details of VMD, see http://www.ks.uiuc.edu/Research/vmd/

Solvent accessible volume on the virus surface was modeled with a spherical probe of 2 Å radius to mimic the size of the linker molecule. Possible probe positions were calculated around the selected sulfur binding site with a distance smaller than 18 Å which corresponds to the length of linker+pMBA complex. Possible cluster core positions at the surface were probed with a sphere of 10 Å radius and the same distance as described above. 10 Å radius was selected because it corresponds to the size of the rigid core structure, including all Au and S atoms, of the Au_{102} cluster.

The possible route for the linker molecule from the sulfur of the cysteine to the virus surface was estimated using all the possible 2 Å probe positions with a following procedure:

1. take one of the closest probes to the cysteine sulfur atom
2. take randomly the next probe position
3. check if the distance of the selected probe to the previous probe is < 2 Å and also that the new probe position is closer to the surface of the virus than the previous
   4. a) if answer to 3. is yes, extend the route with the probe just picked and continue closer to the surface using the same procedure
   4. b) if answer to 3. is no, pick randomly a new probe position until it satisfies the requirements in 3.
5. stop the loop as the last of the selected probes is in contact with one of the possible 10 Å probe at the surface

For all of our analysis we selected the three outermost cysteines that are spatially different enough from each other, namely cys256, cys209 and cys73 for EV1 and cys134, cys234 and cys73 for CVB3. With "spatially different enough" cysteines we mean cysteines of different index that are farther away from each other than the size of the Au_{102} cluster. To clarify the previous we assumed the following: 1) the gold clusters should not overlap as they bind to two adjacent thiol sites, 2) binding occurs most probably to the sulfur that is closer to the surface and has the better accessibility from the two adjacent cysteines, 3) the resolution of TEM-images is not good enough to separate reliably two too closely positioned clusters as two separate spots. To help doing the selection of the most probable cysteine sites we quantitatively compared the solvent accessible volume around the sulfur, which, in addition to the shorter distance from the surface, favors for example cys234 over cys208, cys134 over cys253 and cys73 over the cys208 and cys253 in CVB3.
**Analysis of TEM images of cluster-virus conjugates.** Qualitative analysis of the cluster positions in the measured TEM images was done by comparing the patterns of the spots to the arrangement of the cysteine binding sites in the model structures. Before the analysis, the orientation and the size of the model structure was tuned to have a match with the boundaries of the virus in the corresponding TEM image. Also the orientation of the unique shapes like pentagonal groups of the spots in the TEM image was used for fine-tuning the orientation of the model structure.

To analyse quantitatively the cluster positions in measured TEM images, we calculated the distances of the cysteine binding sites in the model structure. We calculated the distributions of sulfur-sulfur distances of the selected cysteines for both virus models using 5 nearest neighbors for each sulfur-atom and a cutoff distance of 2.5 nm. Selected cutoff distance corresponds roughly to the diameter of one Au$_{102}$(pMBA)$_{44}$ cluster and it neglects the unresolvable and/or non-realistic short distance range from the data as discussed above. For the corresponding analysis of experimental TEM-images we used 3 nearest neighbors for each cluster spot for which the center was determined from the color intensity. For completeness we checked also the results using 5 nearest neighbors in the analysis of the measured TEM-images.
Fig. S1. TEM image of Au$_{102}$(pMBA)$_{44}$ clusters. Note the apparent uniform size. TEM magnification $1 \times 10^5$. 
Fig. S2. $^1$H NMR spectra of Au$_{102}$(pMBA)$_{44}$ in D$_2$O-NaOH at 298K. The spectrum is very similar to the one reported recently by Ackerson and collaborators (SI Ref. 1).
Fig. S3. Optical absorption of functionalized Au102-MI 3 and pure Au102(pMBA)$_{44}$ 2 in H$_2$O. The spectrum of 3 has been scaled to that of 2 below 300 nm where the maleimide linker is not expected to have effect on the spectrum. Significant increase in absorption is observed below 230 nm after the maleimide linkage. Inset shows the difference between spectra 2 and 3 along with the absorption spectra of the pure N-(6-hydroxyhexyl)maleimide molecule 1 in CH$_3$CN.
Fig. S4. 18% PAGE analysis showing (A) $N$-(6-hydroxyhexyl)maleimide functionalized $\text{Au}_{102}(\text{pMBA})_{44}$ clusters and (B) monodisperse $\text{Au}_{102}(\text{pMBA})_{44}$ clusters. The functionalization was confirmed by gel electrophoresis visualization, which showed the covalent attachment of the maleimide linker molecule to the $\text{Au}_{102}(\text{pMBA})_{44}$ (2) cluster. This is seen as multiple bands of higher molecular weight species on the polyacrylamide gel since the number of the reacting maleimide linker molecules on a particular cluster cannot be accurately controlled. However, only the water soluble maleimide decorated $\text{Au}_{102}(\text{pMBA})_{44-m}(N$-(6-hydroxyhexyl)maleimide)$_m$ (3) were collected. Also, the distinctive brown color of the $\text{Au}_{102}$ cluster shows that the gold core of the maleimide decorated gold clusters has remained intact during the covalent attachment of the maleimide ligands on the cluster pMBA surface.
Fig. S5. $^1$H and $^{13}$C NMR spectra of $N$-(hydroxyhexyl)maleimide in CDCl$_3$ at 298K.
Fig. S6. IR absorption spectra of Au102-MI 3, Au102(pMBA)44 2, and N-(6-hydroxyhexyl)maleimide 1. Inclusion of the linker in the clusters gives rise to a new peak at 950 cm⁻¹, attributed to ester group vibrations in benzoates, and broad shoulders at both sides of the benzene ring vibration (1013 cm⁻¹) (SI Ref. 2). The inset shows second-order differential of the spectra, which reveals four new peaks in the functionalized cluster at 1000 cm⁻¹, 1025 cm⁻¹, 1043 cm⁻¹, and 1058 cm⁻¹, corresponding to the hexyl-chain C-C-C skeletal and CH₂ rocking vibrations of the linker molecule (SI Ref. 3). Spectrum of 1 was measured from KBr-tablet, and 2 and 3 were measured from dried film in transmission geometry.
Fig. S7. TEM images of EV1 viruses conjugated with non-functionalized Au₁₀₂(pMBA)₄₄ after different incubation times: 15 min (A), 2h (B), 24h (C), 48h (D). Phosphotungstic acid has been used as staining agent. Single Au₁₀₂(pMBA)₄₄ clusters can be seen but they are not bound to the viruses. The used magnification and other parameters for imaging are shown below each figure.
**Fig. S8.** TEM images of EV1 viruses conjugated with functionalized Au102-MI after different incubation times: 15 min (A), 2h (B), 24h (C), 48h (D). Phosphotungstic acid has been used as staining agent. A few larger aggregates of gold clusters are visible in (C) and (D). The used magnification and other parameters for imaging are shown below each figure.
**Fig. S9.** (A): Inverted color picture of Fig. 3A in the main text, processed with Gaussian smoothening (sigma = 50 px) and contrast enhancement. Cluster centers closest to the extremal points of the icosahedron are marked with A - F. The clusters are arranged in a hexagonal pattern around the virus as predicted by projection of icosahedron to two dimensions along the $C_3$-axis. (B): Simple model of the virus capsid and attached clusters. From the X-ray structure of EV1 the inradius $r_{in}$ (center to face) of the icosahedral virus capsid was determined to be 13.0 nm, and the circumradius $r_{circ}$ (center to tip) was estimated between 15.0 -- 16.5 nm (see Fig. S13 for details). With the addition of Au102-MI with 1.4 nm radius, and assuming both the Au-cluster and virus capsid as rigid bodies with no interpenetration, we get $r_{in} = 14.4$ nm and $r_{circ} = 16.4 -- 17.9$ nm. From (A) we measured $r_{in} = 16.7 \pm 0.6$ nm and $r_{circ} = 19.3 \pm 0.8$ nm, slightly larger than expected. The ratio $R = r_{circ} / r_{in}$ for our model system and measured values are $R = 1.19 \pm 0.06$ and $1.16 \pm 0.07$ respectively, which agrees quite well with the value for a perfect icosahedron $R = 1.258$. This indicates close adherence to icosahedral arrangement of the gold clusters. Slight deviations are caused by the projection not being perfectly along the $C_3$-axis of the icosahedron.
**Fig. S10.** Column purification of Au102-MI / EV1 conjugates as described in Materials and Methods. Top: Graph showing the presence of gold (OD405, scale on the right) and virus (pfu/ml, scale on the left) in each fraction. Representative TEM images of fraction 3 (middle) and 4 (bottom). Fraction 3 contains a higher amount of gold-decorated viruses. A few larger aggregates of gold clusters are visible as well. The used magnification and other parameters for imaging are shown below each figure.
Fig. S11. The same as Fig. S10 but for CVB3.
Fig. S12. The same as Fig. S10 but for the control gold (non-functionalized Au\textsubscript{102}(pMBA)\textsubscript{44}).
**Fig. S13.** Radial distribution of all atoms in the virus capsid of EV1 (blue continuous distribution) and CVB3 (pink continuous distribution). The sharp peaks show the radial distribution of sulfur of all cysteines in EV1 (red peaks) and CVB3 (dark blue peaks) viruses. The radial distance is measured from the center of mass. Five outermost cysteine layers correspond to 1) cys134, 2) cys234, 3) cys253, 4) cys208, 5) cys73 in CVB3 and 1) cys256, 2) cys209, 3) cys73, 4) cys112, 5) cys193 in EV1. Their lateral positions are visualized in Fig. S14. The analysis is made on the basis of the published crystal structures of EV1 in ref. 8 and CVB3 in ref. 9.
Fig. S14. Positions and symmetries of the sulfur atoms of the five outermost cysteines in (A) EV1 and (B) CVB3. For EV1 the three outermost cysteines are directly spatially different enough from each other (see SI text) while for CVB3 there exist two groups \{cys134 (blue), cys253 (red)\} and \{cys234 (cyan), cys208 (black)\} for which the different cysteines are spatially very close to each other. From the mentioned two groups the cysteines closer to surface with better accessibility are expected to bind Au102-MI. The 5-fold symmetry of cys73 in both of the viruses is unique; no other cysteine is positioned similarly. The analysis is made on the basis of the published crystal structures of EV1 in ref. 8 and CVB3 in ref. 9.
Fig. S15. Accessible volume near the cysteines of EV1 shown as a transparent red volume which is drawn using the positions of spherical probes of 2 Å radius at the surface. The position of the gold cluster is depicted as the large brown sphere (spherical probe of 10 Å radius), and the sulfur of cysteine as the small yellow sphere. Positioning of the linker molecule is modelled as described in SI text above and is shown as a continuous path of 2 Å probes with a light blue color. From the two labelled distances $d_{\text{surf}}$ (longer arrow) denotes the distance from the sulfur of cysteine to the surface of closest 10 Å spherical probe while $d_{\text{gap}}$ (shorter arrow) describes how far away from the direct contact the closest 2 Å spherical probe is from the sulfur of the cysteine. Linker molecule with pMBA is represented in a realistic scale next to the modeled structures. The analysis is made on the basis of the published crystal structure of EV1 in ref. 8.
Fig. S16. The same as Fig. S15, but for CVB3. The analysis is made on the basis of the published crystal structure of CVB3 in ref. 9.
SI REFERENCES

