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Negatively Charged Glyconanoparticles Modulate and Stabilize the Secondary Structures of a gp120 V3 Loop Peptide: Toward Fully Synthetic HIV Vaccine Candidates

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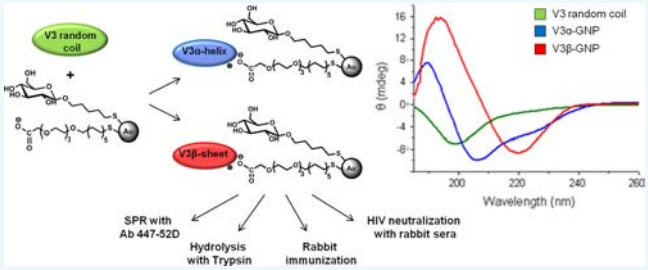
Supporting Information

ABSTRACT: The third variable region (V3 peptide) of the HIV-1 gp120 is a major immunogenic domain of HIV-1. Controlling the formation of the immunologically active conformation is a crucial step to the rational design of fully synthetic candidate vaccines. Herein, we present the modulation and stabilization of either the α-helix or β-strand conformation of the V3 peptide by conjugation to negatively charged gold glyconanoparticles (GNPs). The formation of the secondary structure can be triggered by the variation of the buffer concentration and/or pH as indicated by circular dichoism. The peptide on the GNPs shows increased stability toward peptidase degradation as compared to the free peptide. Moreover, only the V3β-GNPs bind to the anti-V3 human broadly neutralizing mAb 447-52D as demonstrated by surface plasmon resonance (SPR). The strong binding of V3β-GNPs to the 447-52D mAb was the starting point to address its study as immunogen. V3β-GNPs elicit antibodies in rabbits that recognize a recombinant gp120 and the serum displayed low but consistent neutralizing activity. These results open up the way for the design of new fully synthetic HIV vaccine candidates.



INTRODUCTION

Proteins and peptides involved in biological processes adopt a preferential conformation that determines their biological function. However, this conformation is frequently not known and the precise interactions at atomic level that determine the biological activity are not well established. Tools that modulate and control the biologically active conformation of significant epitopes may facilitate the understanding of their interactions and the development of their applications. The merger of chemistry and nanotechnology has been shown to significantly contribute to the development of hybrid materials for biological and biomedical applications. The surface of nanoparticles offers an enormous potential to manipulate the conformation of proteins and peptides. Both positively and negatively charged nanoparticles can stabilize a helical structure in random coil peptides when the charged amino acids are spaced by two or three neutral amino acids.[1,2] Positively charged gold nanoparticles incorporating carbohydrates and amino ligands have also been shown to modulate the presentation of DNA plasmids depending on the configuration of the glycan.[3] Over the years, we have developed sugar coated gold nanoclusters (glyconanoparticles, GNPs) as tools to understand and intervene in carbohydrate mediated processes.[4] Among them, GNPs coated with high-mannose-type oligosaccharides of HIV-1 gp120 were able to inhibit DC-SIGN-mediated HIV-1 *trans*-infection and to interfere with the 2G12/gp120 binding process.[5,6] However, the use of GNPs as platform for other HIV gp120 epitopes has not been addressed so far. We have now focused on the third variable region (V3 peptide) of the HIV-1 envelope protein gp120 (Env) and its multimerization onto GNPs aiming at obtaining fully synthetic HIV candidate vaccines.

The V3 peptide is the third of five variable regions present in gp120. Despite its name, it presents many constant features: a fixed size (30−35 aa), a conserved type II turn at its tip (GPGR/Q), a disulfide bond at its base, a net positive charge,

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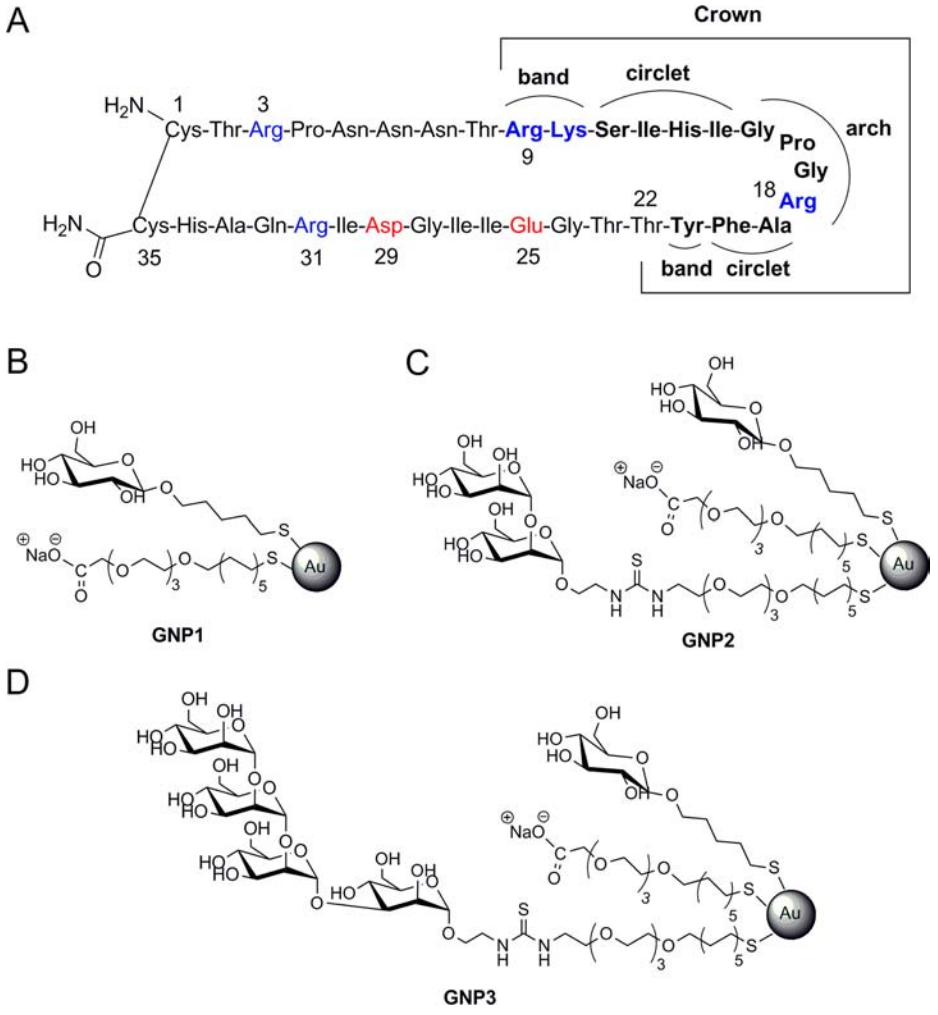

Figure 1. V3 peptide and the negatively charged glyconanoparticles used in this work. (A) Cyclic V3 peptide. Positively charged amino acids are depicted in blue and the negatively charged amino acid in red at neutral pH. (B) GNP1 is covered with 50% of glucose conjugate ($GlcC_5S$) and 50% of carboxyl linker (Linker-CO_2H). (C) GNP2 is covered with 40% of $GlcC_5S$, 50% of Linker-CO_2H, and 10% dimannose conjugate. (D) GNP3 is covered with 40% of $GlcC_5S$, 50% of Linker-CO_2H, and 10% tetramannose conjugate.

and three conserved *N*-linked glycosylation sites within or adjacent to the V3 loop (N295 or N301, N332, and N392).[7] The V3 peptide plays a crucial role in the HIV entry/fusion process as inhibitor of HIV infection.[8−13] Since 1989 it is known that V3 is also highly immunogenic and can be considered a "major neutralizing determinant" of HIV-1.[14] Indeed, anti-V3 antibodies are normally present in almost all HIV-1 infected individuals,[15,16] but the biologically active conformation of the V3 that elicits these Abs remained unknown for several years. The crystal structure of the human IgG broadly neutralizing monoclonal antibody 447-52D in complex with V3 peptide revealed that the V3 amino acid sequence Lys-Arg-Ile-His-Ile (KRIHI) forms an extended β-strand followed by a β turn around the arch (GPGR).[17] The peptide β-strand establishes extensive main chain interactions with the complementary determinant region (CDR H3) of the Ab resulting in a three-stranded mixed β sheet with an up/down/down topology.[18,19] In 2005, the crystal structure of V3 in the complex formed by an HIV-1 gp120 core, a CD4 receptor, and the X5 antibody showed that V3 consists of three different regions: a conserved base, a flexible stem, and a β-hairpin tip.[20] Two years later, the X-ray structure of a tyrosine-sulfated Ab (412d) in complex with gp120 and CD4 was solved and confirmed the presence of a β-strand region that interacts with the Ab.[21] More recently, a novel human broadly neutralizing antibody (PGT 128), capable of recognizing two different $Man_{8/9}GlcNAc_2$ glycans (N301 and N332), as well as the C-terminal of the V3 loop, was isolated.[22] Co-crystallization of PGT 128 Fab with a glycosylated gp120 outer domain containing a truncated V3 loop showed that the V3 loop adopts a β-strand conformation in the complex with PGT 128. This further supports that the β-strand conformation is the biologically active conformation.

Because of the conformational flexibility, the V3 peptide induces a broad distribution of antibodies. Constrained V3 peptides were conjugated to immunostimulant proteins,[23−26] and the multivalent presentation of V3 on different platforms[27−29] has also been explored to increase the immunogenicity. Those results showed that these constructs are able to induce an immune response in animals, but the sera neutralize only T-cell line adapted virus or a narrow range of primary isolates. The reason for this insufficient immune response might be that the V3 peptide on these platforms does not adopt the active conformation which is essential for the interaction with anti-V3 Abs and probably with CCR5 co-receptor.

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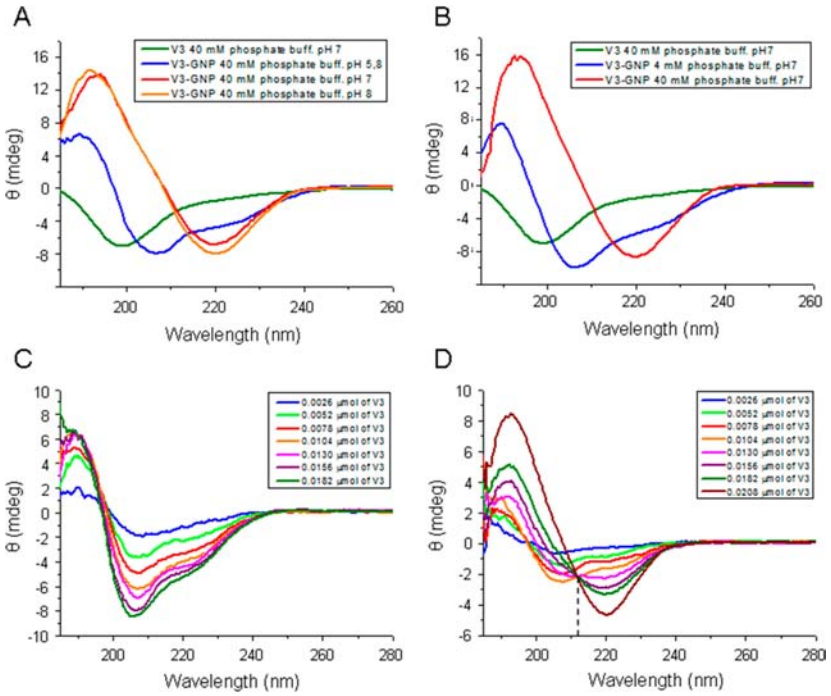

Figure 2. CD spectra of V3-GNPs prepared in different conditions. (A) CD spectra of the free V3 peptide (green) and the V3-GNP1 prepared in 40 mM phosphate at different pH: 5.8 (blue), 7 (red), and 8 (orange). (B) CD spectra of V3-GNPs prepared at pH 7 and two different phosphate buffer concentrations (4 and 40 mM). (C) GNP1 (0.00375 μmol, 0.0125 mM) was titrated in phosphate buffer (4 mM, pH 7) with V3 peptide (7 × 0.0026 μmol). (D) GNP1 (0.00375 μmol, 0.0125 mM) was titrated in phosphate buffer (40 mM, pH 7) with V3 peptide (8 × 0.0026 μmol). From the 0.0104 μM peptide concentration on, an isodichroic point at 212 nm is observed (dotted line). The time between each recorded spectrum is 1 h.

Unfortunately, elicitation of broadly neutralizing antibodies with vaccine constructs seems to be a particularly difficult task.

Gold glyconanoparticles (GNPs) have been shown to have a great potential as vaccine vectors. For example, GNPs incorporating the tetrasaccharide epitope of the capsular polysaccharide of *Streptococcus pneumonia* serotype 14 and a $CD4^+$-restricted peptide generated opsonogenic antibodies in mice[30] or galactofuranose-coated gold nanoparticles elicit a proinflammatory response in human monocyte-derived dendritic cells.[31] We have also approached the design and development of HIV-fully synthetic vaccine candidates by using GNPs as a platform to conjugate gp120 high-mannose-type glycans and immunogenic peptides in a control way.

In this work, we report the results of our investigations to constrain and stabilize the V3 peptide conformation by conjugation to negatively charged gold glyconanoparticles (GNPs). Controlling the formation of the immunologically active conformation is a crucial step to the rational design of fully synthetic candidate vaccines. We investigated the conformational changes of the V3 onto the GNPs by circular dichroism (CD) and the stability toward peptidase degradation by HPLC-mass spectrometry. The interaction of the V3-GNP constructs with a human broadly neutralizing antibody was evaluated by surface plasmon resonance (SPR). The ultimate goal is to obtain V3-GNP constructs with an immunologically active β-strand (V3β-GNP) conformation. Consequently, immunization experiments in rabbits were performed with the V3-GNP constructs.

RESULTS AND DISCUSSION

In Figure 1, the V3 peptide and the GNPs used in this study are depicted. The positive charged V3 peptide is a 35-amino-acid cyclic peptide (MW 3895.3) containing all the conserved elements (bands, circlets, and arch) (Figure 1A) determined by Zolla-Pazner's group.[32] The peptide, with isoelectric point (IP) 9.54, has a net positive charge at neutral pH due to the presence of 4 arginines and 1 lysine. As a platform to multimerize the V3 peptide, we selected three different negatively charged gold glyconanoparticles (GNP1, GNP2, and GNP3) bearing the glycoconjugate 5-mercaptopentyl β-D-glucopyranoside ($GlcC_5S$) and a carboxyl-ending linker (Linker-CO_2H) (Figure 1B,C,D). GNP1 is covered with 50% density of the $GlcC_5S$ conjugate and 50% of the carboxyl ligand (Linker-CO_2H). The GNP2 and GNP3 are covered with 40% of $GlcC_5S$, 50% of Linker-CO_2H, and 10% of the terminal dimannoside (Manα1−2Manα) or tetramannoside (Manα1−2Manα1−2Manα1−3Man) of the gp120 high-mannose-type glycans, respectively, that have been shown to interact with the bnAb PGT128.[22] Glyconanoparticles GNP1, GNP2, and GNP3 have been prepared and characterized as previously described (see Supporting Information).[33,34] The average diameter of the gold core is 2 nm as determined by TEM.

The conjugation of V3 to the GNPs was studied under different conditions and the conformational changes of V3 before and after the conjugation was followed by circular dichroism spectroscopy (CD). Proteins that contain α-helical structure present CD spectra with negative bands at 222 and 208 nm and a positive band at 193 nm, whereas proteins with well-defined antiparallel β-strand show negative bands at 218 nm and positive bands at 195 nm; disordered proteins (random coil) have very low ellipticity above 210 nm and a negative band around 197 nm.[35]

Preparation and Characterization of V3-GNPs. V3-GNPs were prepared in phosphate buffer (4 or 40 mM) at different pH (from 5.8 to 8). In a typical preparation, GNPs (100 μL, 2 mg/mL in H_2O) were added to 100 μL of

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phosphate buffer solution (10 mM or 100 mM) and V3 peptide (55 μL, 2 mg/mL in H_2O) was added to obtain a final phosphate concentration of 4 or 40 mM. The brown solution was left under shaking overnight at RT. No precipitate was detected. The mixture was diluted with mQ water and centrifuged on Amicon filter (30 kDa MWCO) for 5 min at 3000 rpm and 10 °C. The filtrates were collected and analyzed by Bradford test.[36] No peptide was found in the filtrates, indicating that all V3 peptides added remain bound to the GNPs (an average of 6 V3 peptide molecules per GNPs).

The V3-GNPs residue in the filter was dissolved in water, lyophilized, and characterized by ^{1}H NMR, TEM, and agarose gel electrophoresis (see Supporting Information). After the conjugation of the peptide, the size of the gold cluster remained unchanged (2 nm) and no tendency toward aggregation was observed by TEM. Comparison of ^{1}H NMR spectra of the V3 peptide, GNP1, and V3-GNP1 showed that the broad signal at 0.9 ppm (Figure S1A) could be assigned to V3 methyl protons confirming the presence of V3 peptide on GNP1. The remaining protons (with lower peak intensity) could not be assigned due to the broadening of the peptide signals onto the GNPs. The agarose gel showed that GNP1 with and without V3 had almost the same retention time (Figure S1B), but after staining with blue Coomassie, only the V3-GNP1 exhibited the typical blue color due to the presence of the peptide (Figure S1C). The presence of the peptide did not significantly change the negative charge of the GNPs at the experimental pH (8.3) as confirmed by the ζ-potential data (Figure S2).

The interaction of the negatively charged GNPs with the positively charged V3 peptide induced a conformational change for V3 which was shown to depend on the concentration and the pH of the phosphate buffer as determined by CD (Figure S3). The conformation shifted from random coil (green line) to predominant α-helix (V3α-GNP, blue line) or β-strand (V3β-GNP, red, and orange line) after binding to GNP (Figure 2). However, the conformation of V3 free peptide does not depend on either the peptide or the phosphate concentration (Figure S4).

The formation of the V3α- or V3β- conformation onto the GNPs seems to depend on the peptide charge and not on the GNP one. The negative charge of the GNPs remains nearly constant between pH 6−8 (−15 to −20 mV) as indicated by the ζ-potential data (Figure 2S), whereas the charge of the peptide significantly changes with the pH. At pH 5.8 all basic amino acid side chains of the V3 (isoelectric point 9.54), including histidines (pK_a = 6), are protonated and can interact with the carboxylic groups of the GNPs. The formation of V3α-GNP1 can be explained by the interaction of the positively charged amino acid sequence **KSIHIGPGR** (circle and arch, Figure 1A) of V3 with GNPs. A similar feature was reported by Lundqvist et al.,[2] in the case of a peptide, displaying positively charged amino acids spaced by two or three neutral amino acids, which exhibited an α-helix conformation upon its interaction with negatively charged silica nanoparticles. The formation of the V3β-strand took place only at high concentration of buffer (40 mM) and above pH 7 (Figure 2A). At pH > 7, the phosphate has two negative charges and it can be speculated that the phosphate dianions aggregate and reorganize the peptide conformation resulting in the V3β-GNP1. The formation of V3β-GNP1 is not only concentration- but also time-dependent. Titration of a GNP1 solution in 4 mM phosphate buffer (pH 7) with increasing concentration of V3 peptide (10 μL, 1 mg/mL in water) at RT for 8 h resulted in a stable α-helix conformation that remains stable over the time (Figure 2C). When the titration is carried out in 40 mM phosphate solution (pH 7), the formation of the α-helix was first observed. However, following four additions of peptide (0.0104 μmol, 4 h), the α-helix converted into a stable β-strand (Figure 2D) with an isodichroic point at 212 nm. A similar isodichroic point (211 nm) was observed by Zhang et al.[37] for the abrupt structural transition of a peptide from a β-sheet to a stable α-helix conformation by increasing temperature.

We also carried out an experiment where the total peptide concentration was added at once to the GNP1 solution either under the conditions of α-helix (0.0182 μmol, buffer 4 mM, pH 7) or β-sheet formation (0.0208 μmol, buffer 40 mM, pH 7). The changes in the CD spectra were recorded after 1 and 15 h from the addition. In the conditions of V3α-GNP1 formation, no changes were detected in the CD spectra over time (Figure S5A). In contrast, in the conditions of V3β-GNP1 formation the α-helix was first observed, but after 15 h only the stable β-strand conformation was formed (Figure S5B).

To demonstrate that it is the phosphate concentration which drives the formation of the β-strand conformation and not the ionic strength of the solution, the peptide was added to a solution of GNP1 in 4 mM phosphate buffer (pH 7) containing NaCl 80 mM (the ionic strength corresponds to 35 mM phosphate buffer, pH 7). The CD spectrum obtained was completely different from the V3α-GNP1 or V3β-GNP1 one and showed a weak minimum at 218 nm (Figure S6). This indicates that the concentration of phosphate is essential to induce and stabilize the peptide in the β-strand conformation. All these results indicate that the positively charged V3 peptide onto the negatively charged nanoplatform can adopt two different stable conformations under controlled experimental conditions.

Both the V3α-GNP1 and V3β-GNP1 once formed remained stable under purification and lyophilization conditions. The stability of the V3α- and V3β-conformations on the GNPs was further assessed by recording the CD spectra of V3α-GNP1 and V3β-GNP1 solutions (1 mg/mL) in phosphate buffer 5 mM at different pHs (3 to 9) and temperatures (10 to 70 °C). Only slight changes in the CD spectra of both GNPs were detected at the highest temperature (Figure S7). By changing the pH, no changes were observed in the CD spectra between pH 5 and 9 of both V3α- and V3β-GNPs. When the pH was decreased to 3, a black precipitate was formed and the CD spectra of the filtered solutions revealed the presence of V3 free peptide in a random coil conformation (Figure S8, purple line). At this pH, the carboxylic groups of the GNPs are protonated and the electrostatic interactions with the positively charged V3 peptide disappear. GNPs negative charges are necessary to modulate and stabilize the peptide in the α-helix or β-strand conformation. Indeed, the incubation of the V3 peptide with GNPs bearing only the glucose linker ($GlcC_5S$) did not change the random coil conformation as indicated by the CD spectra (data not shown).

The V3β-GNP2 and V3β-GNP3 incorporating the dimannoside Manα1−2Manα and the tetramannoside Manα1−2Manα1−2Manα1−3Man (Figure 1) were prepared under the same conditions as those for the preparation of V3β-GNP1 (6 equiv V3, 40 mM phosphate buffer, pH 7). The resulting CD spectra of V3β-GNP2 and V3β-GNP3 were similar to that of V3β-GNP1 except for a slight shift toward higher wavelengths (Figure S9). This small difference between the CD spectra may indicate the occurrence of a possible

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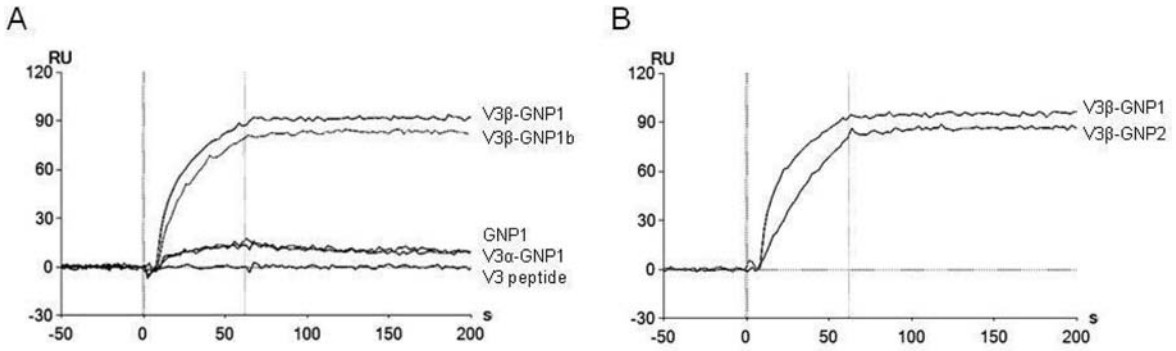

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	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	Rmax	Chi2
GNP1	$6.09\ 10^{4}$	$2.13\ 10^{-3}$	9.15	1.75
V3α-GNP1	$4.52\ 10^{4}$	$1.82\ 10^{-3}$	10.5	1.35
V3β-GNP1	$3.02\ 10^{4}$	$1.19\ 10^{-16}$	97.1	3.72
V3β-GNP1b	$2.91\ 10^{4}$	$3.94\ 10^{-18}$	95.8	4.71
V3β-GNP2	$1.15\ 10^{4}$	$2.64\ 10^{-17}$	94.5	6.35

Figure 3. SPR experiments. mAb 447-52D was coupled to Proteon sensor chip using the recommended procedure until final 250 RU. (A) Sensograms show the binding of V3 peptide (0.05 mg/mL) and GNP1, V3β-GNP1, V3β-GNP1b, and V3α-GNP1 (0.2 mg/mL) to mAb 447-52D. (B) Sensograms show the binding of V3β-GNP1 and V3β-GNP2 (0.2 mg/mL) to mAb 447-52D. (C) Table with the rate binding constants of GNP1, V3α-GNP1, V3β-GNP1, V3β-GNP1b, and V3β-GNP2 to anti-V3 human bnAb 447-52D.

interaction between the mannose oligosaccharides and V3 peptides.

Interaction Studies of V3-GNPs with the Anti-V3 Human bnAb 447-52D by SPR. The selectivity of the α-helix and β-strand conformations bound to GNP in the recognition of immune epitopes was evaluated. To do so, the interaction of the V3-GNPs with the human broadly neutralizing anti-V3 Ab 447-52D, specific for the conserved tip of the V3 loop,[17] was tested by surface plasmon resonance (SPR). Standard amine coupling method was used to immobilize the Ab 447-52D on research-grade GLC Proteon sensor chip until 250 resonance units (RU). The binding of each analyte, control GNP1, free V3 peptide, V3α-GNP1, V3β-GNP1, V3β-GNP1b (with double amount of V3), and V3β-GNP2 (with the dimannoside), to Ab 447-52D was studied at six different concentrations (0.2, 0.1, 0.05, 0.025, 0.0125, and 0.00625 mg/mL in PBS/Tween buffer). The binding profiles (sensorgrams) were obtained by automatic subtraction of the reference surface signals from the 447-52D-activated surface signals (Figure S10). The channel surface between each runs was regenerated with a short pulse of 0.1 M HCl. In all cases (except for V3 peptide) binding to the Ab occurs in a concentration dependent manner (Figure S10). The strongest binding (highest RU) was obtained for the GNPs containing the V3 in the β-strand conformations (Figure S10A,B,D), whereas the V3α-GNP1 displays a very weak binding similar to the control GNP1. Sensograms corresponding to the highest tested concentration of V3β-GNP1, V3α-GNP1, GNP1, and V3 peptide were grouped under the same representation (Figure 3A) for the sake of comparison.

Only V3β-GNPs bind strongly to Ab 447-52D in agreement with the crystallographic structure of the V3 peptide in complex with Ab 447-52D, where V3 peptides bind the Ab with a β-strand conformation.[17] The low affinity of V3α-GNP1 and GNP1 for the antibody is the same and probably due to nonspecific electrostatic interactions. The binding of the free V3 peptide to the Ab was not detected despite the high concentrations used relative to the concentration of V3 on the GNPs. This result can be attributed to the random coil conformation of the free peptide in solution. The difference between the specific interaction of V3β-GNP1 and the unspecific of V3α-GNPs may lie in the accessibility of V3 arch (GPGR) in the V3β-GNP1 due to the adopted conformation. GNPs with double amount of V3 (V3β-GNP1b, 12 V3 per GNP1) were also tested. V3β-GNP1b is able to bind to the Ab without exhibiting any enhanced interaction (Figure 3A and Figure S10B). This can be explained by the fact that each well-oriented antibody on the chip can bind two V3 peptides, and an excess of V3 peptides does not enhance the binding. The binding of V3β-GNP2 bearing Manα1−2Manα disaccharides and V3 peptides with β-strand conformation showed similar affinity for the Ab 447-52D than the one observed for V3β-GNP1 (Figure 3B). No binding was observed for the GNP2 lacking the V3 peptides (data not shown). The kinetic binding constants (k_{on} and k_{off}) obtained from each group of 6 sensograms (Figure S10) are displayed in Figure 3C. The association constant, k_{on}, for GNP1, V3α-GNP1, V3β-GNP1, and V3β-GNP1b has similar values (around 10^4). However, the dissociation constant, k_{off}, is much lower for V3-GNP with β-strand conformation, indicating a stronger binding.

In conclusion, the SPR binding study showed that only GNPs bearing V3 peptide in β-strand conformation (V3β-GNPs) are able to interact strongly with Ab 447-52D. GNP1 and V3α-GNP1 bind weakly to the Ab. The strong binding of V3β-GNPs to the broadly neutralizing 447-52D Ab is a starting point to address the study of V3-GNPs as immunogens.

Stability of V3β-GNP1 to Trypsin Hydrolysis. Peptides are not used as immunogens because of their low stability against enzymatic hydrolysis of peptidases contained in blood. To demonstrate that the GNPs may protect the V3 peptide against peptidases, the enzymatic hydrolysis by trypsin of the V3 free peptide and the V3β-GNP1 were compared. Trypsin is

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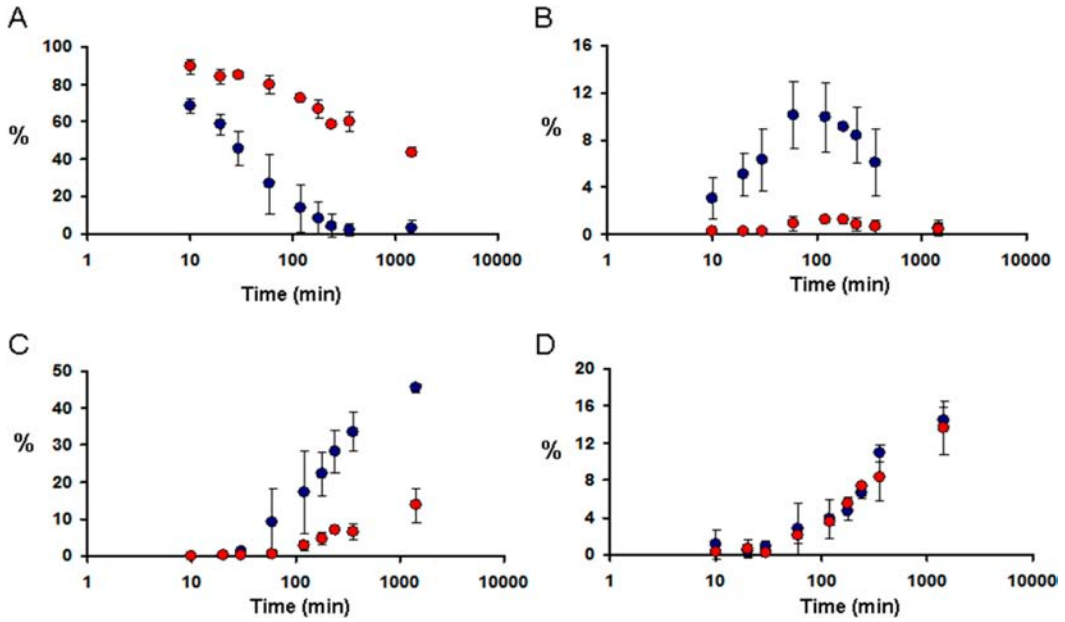

Figure 4. HPLC-MS data of V3β-GNP1 (red) and of V3 free peptide (blue) hydrolysis by trypsin. Trypsin (10 μL, 0.005 mg/mL) was added to a solution of V3 peptide (1 mL, 0.05 mM) and to a solution of V3β-GNP1 (1 mL, 0.05 mM in V3 peptide) in phosphate buffer (50 mM, pH 7.6). The kinetics of the hydrolysis was followed by HPLC-MS during 24 h. (A) Percentage of complete V3 peptide. (B) V3 peptide with one hydrolysis. (C) V3 fragments 10−18: KSIHIGPGR. (D) V3 fragments 19−31: AFYTTGEIIGDIR.

a protease able to hydrolyze the amidic bond at the carboxyl side of lysine and arginine, except when they are followed by proline. The V3 peptide used in this work contains 4 possible hydrolyzable trypsin amidic bonds. Enzymatic degradation was performed using the same amount of V3 in the free form and bonded to GNP. Trypsin (10 μL, 0.005 mg/mL) was added to a solution of V3 peptide (1 mL, 0.05 mM) and to a solution of V3β-GNP1 (1 mL, 0.05 mM in V3 peptide) in phosphate buffer (50 mM, pH 7.6). The kinetics of enzymatic hydrolysis was followed by HPLC-MS during 24 h. After chromatographic separation by UPLC, the V3 peptide and the trypsin digested fragments were identified and assigned by ESI-MS. The relative quantification was performed using the integrated peak areas obtained by UV detector (λ 220 nm). The percentage of the complete V3 peptide (without any hydrolysis) and the most relevant V3 fragments was determined after 10 min, 20 min, 30 min, 1 h, 2 h, 3 h, 4 h, 6 h, and 24 h. The most significant kinetics of V3 peptide fragmentation are showed in Figure 4 and Figure S11.

The percentage of non-hydrolyzed V3 peptide (MW 3895.3) as a function of time is shown in Figure 4A. The V3 free peptide disappeared completely (blue points) after 6 h, while the peptide on the GNP underwent only 40% of hydrolysis (red points) in the same time. The formation of the first V3 fragment (MW 3913.3; V3 + H_2O) and its subsequent hydrolysis (Figure 4B and Figure S11A) indicates a higher stability of the peptide on the GNP (red points) as compared to the free peptide (blue points). The percentage of the KSIHIGPGR (aa 10−18) fragment, resulting from two amide cleavages (Figure 4C) is 4 times higher for the V3 free peptide than for the V3β-GNP1. The kinetics of the KSIHIGPGR formation is very similar to that of the SIHIGPGR (aa 11−18) fragment (Figure S11B). In contrast, the percentage of the AFYTTGEIIGDIR (aa 19−31) fragment is the same for the V3 free peptide and the V3β-GNP1 (Figure 4D). This means that this fragment has the same exposure when bound to the GNP as in the free peptide. Otherwise, the most stable region to the trypsin hydrolysis should be the one that interacts with the nanoparticle. This region corresponds to the amino acids 10−18 (KSIHIGPGR) because of it higher percentage in the hydrolysis of the free V3 peptide in comparison to that of the V3 on the GNP1.

In an attempt to support these findings, the spatial distribution of electrostatic charges for different conformations of V3 peptide was examined (Figure S12). Electrostatic potential was calculated using the Adaptative Poisson−Boltzmann Solver (APBS) tools as implemented in the *Pymol* software (www.pymol.org). Electrostatic potential was mapped on the solvent accessible surface; a blue color indicates regions of positive potential whereas red depicts negative potential values. Figure 5A represents the V3 peptide with a β-strand conformation as determined from the X-ray structure of gp120 in complex with CD4 and X5 Ab (PDB 2B4C).[20] Figure 5B shows the V3 peptide with a β-strand conformation as determined from the X-ray structure of gp120 in complex with CD4 and the anti-V3 Ab 412d (PDB 2QAD).[21]

Both structures A and B (Figure 5) could interact with the negatively charged GNP1. Nevertheless, the concave form of the positive surface in conformation B and the fact that to interact selectively with the 447-52D Ab the V3β-GNP1 should have access to the V3 arch (GPGR) would indicate that conformation B is the preferred one for binding to the GNPs. This conformation leaves the amino acid sequence AFYTTGEIIGDIR (aa 19−31) more accessible for trypsin hydrolysis, a feature which agrees with the experimental results obtained by HPLC-MS analysis.

V3-GNPs as Immunogens. Immunogenicity of peptides is usually very poor and may be attributed to such features as conformational flexibility, difficulty in reproducing the conformational restraint of protein epitope, and the propensity to undergo proteolysis in vivo. Coupling to highly immunogenic proteins is a general strategy to enhance the humoral immune

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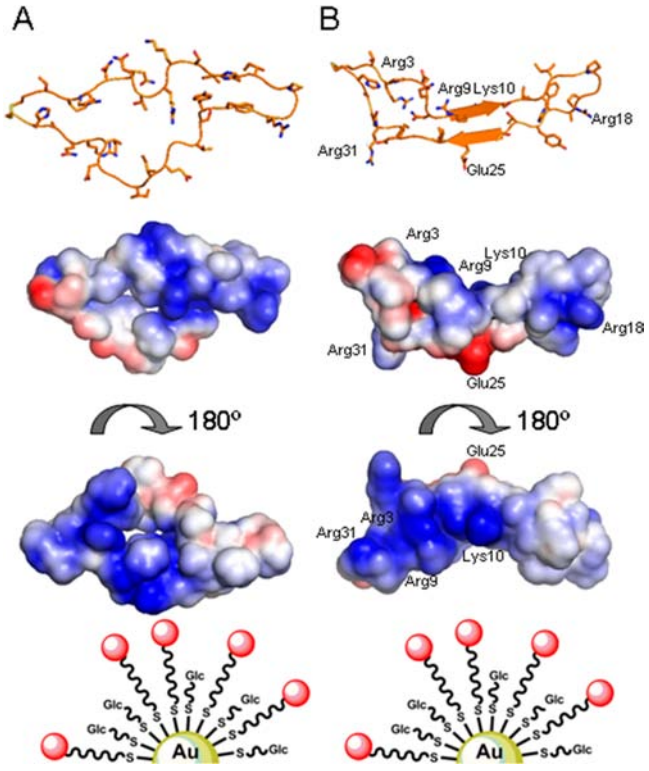

Figure 5. Representation of different V3 structures. (A) V3 peptide with a β-strand conformation determined by X-ray structure of gp120 complexed with CD4 and X5 Ab (PDB 2B4C).[20] (B) V3 peptide with a β-strand conformation determined by X-ray structure (5 nm) of gp120 complexed with CD4 and the anti V3 Ab 412d (PDB 2QAD).[21] Electrostatic potential was calculated using the Adaptative Poisson–Boltzmann Solver (APBS) tools as implemented in the *Pymol* software. From X-ray V3 peptide is 50 Å long. a GNP cartoon was prepared taking into account TEM data and a theoretical calculation of the length of the linker. The GNP diameter was estimated to be around 5 nm.

response of peptides. However, the coupling of the peptide to the carrier protein does not always increase the immune response because the peptide may be not accessible or may assume an unpredictable, inactive conformation.

GNPs offer an alternative way to conjugate peptides and restrain their conformational flexibility. It has been previously shown that a fully synthetic carbohydrate vaccine, based on gold nanoparticles incorporating the repeating tetrasaccharide of the *Streptococcus pneumonia* capsular polysaccharide and a T-helper peptide was able to evoke anti-carbohydrate antibodies in mice that opsonize the bacteria in spleen infected cells.[30] Encouraged by this positive result and supported by the SPR results with Ab 447-52D, we expected that V3β-GNPs could elicit anti-V3 Abs in in vivo experiments. Immunization was performed with GNP1, V3β-GNP1, and V3β-GNP3 using five female New Zealand White rabbits for each sample. Three injections of nanoparticles were performed: one prime and two boosts. The prime was performed with 60 mg of nanoparticles and adjuvants. After 34 days of injection, rabbits were boosted with 50 μg of nanoparticles and blood was taken after 8 and 12 days from the boost (B1–8 and B12). After 34 days from the first boost, the second and last boost was performed with 50 μg of nanoparticles and blood was taken after 8 and 12 days from the boost.

All rabbit sera were tested by GNP-ELISA test[38] where the antigen on the ELISA plate was the nanoparticles that generate the corresponding sera (Figure 6A and Figure S13A and C). ELISA was also performed with a recombinant gp120 as antigen (Figure 6B and Figure S13B and D). Almost all IgGs contained in rabbit sera immunized with V3β-GNP1 are able to bind V3β-GNP1 on ELISA plate (Figure 6A). Three of these sera (sera of rabbits 2, 4, and 5) are also able to bind the recombinant gp120 (Figure 6B).

HIV Neutralization Assays. The sera that showed the best binding to gp120 (Figure 6B, rabbit 2, and Figure S13D, rabbit 13) were tested in HIV neutralization experiments. In this assay, a recombinant R5 tropic HIV-1 (JR-renilla) was used to infect susceptible cells (U87-CCR5). After 45 min of incubation with the effective dilutions of sera antibodies, viral supernatants were used to infect cell cultures. Only sera coming from rabbit immunized with V3β-GNP1 showed a slight non-dilution-dependent inhibition of HIV infection (~30%), as compared to a nontreated control (Figure 7). Preimmune sera did not inhibit infection at all. Inhibition with the other GNP-elicited sera (V3β-GNP3) was not significant.

Antibodies against HIV are easily generated, but their efficacy to block viral infection is much harder to achieve. HIV has several ways to escape antibody activity such as hidden neutralizing domains, high variability, and different patterns of glycosylation or epitopic masking. Beside, full neutralization has only been achieved either by a combination of antibodies targeting different envelope domains or by highly specific monoclonal antibodies with strong affinity that are generated in rare patients after a long process of B-cell maturation.[39] The antibodies generated by rabbit immunization were able to bind to HIV-1 gp120, but as expected, their efficacy as neutralizing

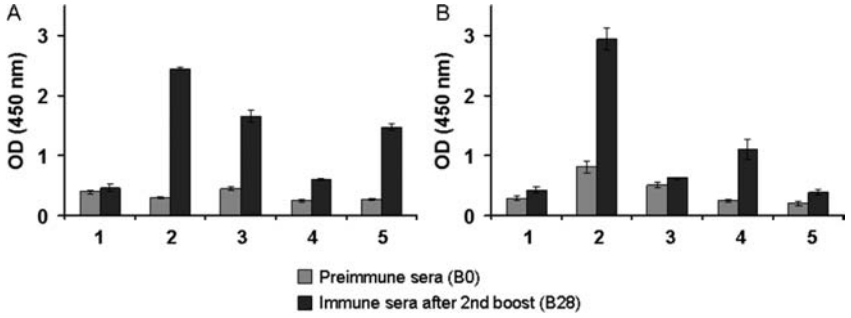

Figure 6. ELISA of IgGs from rabbit immunized with V3β-GNP1. All sera were diluted 1:100 with PBS. IgGs sera were detected by HRP-goat anti-rabbit (450 nm). (A) Binding of rabbit sera (rabbits 1–5) to V3β-GNP1 by GNP-ELISA. A clear and significant IgGs response of B28 sera against V3β-GNP1 was detected in almost all rabbits. (B) Binding of rabbit sera IgGs (1–5) to gp120 by ELISA.

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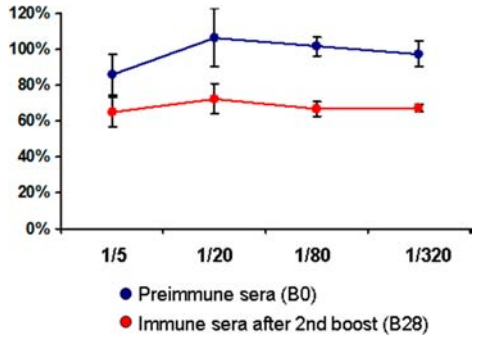

Figure 7. HIV-1 neutralization assay with V3β-GNP1. JR-Renilla HIV virus was preincubated for 45 min with B28 sera (second boost) and afterward used to infect U87-CCR5 cells. RLUs were obtained 48 h postinfection measuring renilla-luciferase activity in lysed cell cultures in a luminometer. Nontreated viral supernatants were considered as 100% of infection.

antibodies was low. One possible reason for low neutralization may be that the lack of glycosylation as HIV-1 neutralization by anti-V3 antibodies is partially dependent on appropriate glycosylation of critical residues.[40] This may be the reason the neutralization with the V3β-GNP3 was not significant. Nevertheless, the V3 domain in the gp120 represents a preferential target for the induction of neutralizing antibodies as it is considered an "Achilles heel" of HIV-1. Moreover, antibodies targeting V3 regions have been isolated from several broad neutralizer patients.[41,42]

The efficacy of the V3β-GNP1 against the R5 tropic HIV-1 has scarcely reached 30%, but despite this limitation the simple fully synthetic V3β-GNP1 construct is capable to generate antibodies in rabbits without any assistance of highly immunogenic proteins. The antibodies bind to HIV-1gp120 and the serum displayed low but consistent neutralizing activity. Therefore, V3β-GNP1 may be considered a starting point to design new vaccine candidates with improved neutralizing potency to achieve strong neutralizing activity of elicited antibodies.

CONCLUSIONS

We demonstrate that by using negatively charged GNPs the conformation of the V3 peptide can be modulated to obtain either stable α-helix (V3α-GNP) or β-strand (V3β-GNP) conformation. The peptide on the GNPs showed increased stability toward peptidase degradation as compared to the free peptide. Moreover, the selectivity of the V3β-GNP versus the V3α-GNP for the anti-V3 human broadly neutralizing mAb 447-52D was demonstrated by surface plasmon resonance (SPR). Finally, immunization experiments in rabbits showed that the fully synthetic GNP-constructs are capable of eliciting an immune response even in the absence of highly immunogenic proteins. All these results support our strategy for the development of fully synthetic HIV candidate vaccines based on GNPs and immunogenic epitopes.

EXPERIMENTAL PROCEDURES

General Methods and Instruments. General methods and instruments are described in the Supporting Information.

General Procedure for the Preparation of the GNPs. Briefly, an aqueous solution of tetrachloroauric acid was added to a MeOH/H_2O/AcOH solution of a mixture of thiol ending molecules. In the case of GNP1, $GlcC_5$ and Linker-CO_2H were mixed in 1:1 proportion; GNP2 was prepared using a mixture of $GlcC_5$:Linker-CO_2H:Manα1−2Man in the ratio 4:5:1. Each mixture was reduced with an excess of $NaBH_4$ and the suspension was vigorously shaken for 2 h at room temperature (RT). The supernatant was removed, the residue was washed three times with EtOH and/or MetOH and finally dissolved in Milli-Q water, purified by dialysis, and characterized by ^1H NMR spectroscopy, transmission electron microscopy (TEM), and ultraviolet spectroscopy (UV). GNPs obtained were water-soluble and well dispersed with a gold nucleus diameter of around 2 nm as found by TEM.

General Procedure for the Preparation of the V3-GNPs. A water solution of GNP (1 mL, 2 mg/mL, 0.025 μmol) was mixed with a phosphate buffer solution (1 mL, 10−100 mM, pH 5.8−9) and a water solution of V3 peptide (0.55 mL, 1 mg/mL, 0.14 μmol) was added. The brown solution was left under shaking overnight at RT. The mixture was diluted with 2.55 mL of distilled water and centrifuged on amicon filter (30 kDa MWCO) for 5 min at 3000 rpm and 10 °C. The residue and the washing were collected. The V3-GNP was lyophilized and the washing analyzed with Bradford test.

Gel Electrophoresis. Agarose gel electrophoresis of GNP1 and V3β-GNP1 was performed with Tris-Borate EDTA (88 mM Tris Base, 88 mM boric acid, and 2 mM EDTA) as running buffer (Figure S1). GNP1 and V3β-GNP1 (2 mg/mL in H_2O) were mixed with 4 μL of TBE (5×) and 2 μL of glycerol (30%) and loaded in the gel. The gel was run at 50 V for 1 h (running buffer TBE 0.5×). The gel was stained for 1 h at room temperature with gentle shaking with SimplyBlue SafeStain (Invitrogen).

ζ-Potential Experiments. All ζ-potential measurements were performed using a MALVERN Zetasizer Nano ZS. GNPs (100 μg/mL) were tested in phosphate buffer pH 6, pH 7 and pH 8 at 25 °C. Three rounds of assays were conducted and the average data are reported.

Circular Dichroism (CD) Experiments. Experiments were performed on a Jasco J815 spectrophotometer using a quartz cuvette with a 1 mm path length. For all CD studies, scans were taken from 180 to 280 nm at a rate of 20 nm/min with a sample interval of 0.1 nm and an 8 s response. Each experiment gives a final spectrum which is the average of ten scans. All CD spectra were subtracted from blank.

SPR Experiments. The binding of GNPs and V3 peptide (in here, analytes) to mAb 447-52D was studied by SPR using a ProteOn XPR36 Protein Interaction Array System with research-grade GLC sensor chips (compact polymer layer containing easily activated carboxylic groups). mAb 447-52D was immobilized on a GLC sensor chip using the standard amine coupling chemistry according to the manufacturer's instructions. A GLC sensor chip was equilibrated with ProteOn PBS/Tween buffer (phosphate buffered saline, pH 7.4, 0.005% Tween 20). The carboxylic groups of two different channels on the chip surface were activated at 25 °C by injecting 30 μL (contact time: 60 s; flow rate: 30 μL/min) of a 1 vol/vol mixture of EDAC (16 mM) and Sulfo-NHS (4 mM). Channel 1 was further injected with 120 μL (240 s; 30 μL/min) of a 447-52D solution in acetate buffer (50 μg/mL, 10 mM, pH 4) at 25 °C to obtain an immobilization level of 250 Response Units (RU). Only PBS/Tween was flowed into channel 2, which was used as a reference. Finally, the surfaces of both channels were saturated by 100 μL (200 s; 30 μL/min) of ethanolamine HCl (1 M, pH 8.5). Simultaneous binding of six different concentrations of each analyte to 447-52D was

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studied. Each analyte was diluted at 0.2, 0.1, 0.05, 0.025, 0.0125, and 0.00625 mg/mL in PBS/Tween buffer, and injected at 25 °C in both channels (contact time: 60 s; dissociation time: 100 s; flow rate: 100 μL/min). The binding profiles (sensorgrams) were obtained by an automatic subtraction of the reference surface signals from the 447-52D-activated surface signals. The sensor surface between runs was regenerated with a short pulse of 0.1 M HCl. All binding experiments were repeated three times and the binding RUs were comparable, taking into account the instrument error.

Trypsin Hydrolysis. Trypsin (10 μL, 0.005 mg/mL) was added to a solution of V3 peptide (1 mL, 0.05 mM) or to a solution of V3β-GNP1 (1 mL, 0.05 mM in V3 peptide) in phosphate buffer (50 mM, pH 7.6). Enzymatic hydrolysis was followed by HPLC-MS during 24 h. The percentage of the complete V3 peptide (without any hydrolysis) and the most relevant V3 fragments was determined after 10 min, 20 min, 30 min, 1 h, 2 h, 3 h, 4 h, 6 h, and 24 h. Each aliquot (100 μL) was quenched with 600 μL of HCl (0.1 N), lyophilized, and resuspended with 70 μL of HPLC H_2O before HPLC-MS analysis. After chromatographic separation by UPLC, the V3 peptide and the trypsin digested fragments were identified and assigned by ESI-MS. The relative quantification was performed using the integrated peak areas obtained by UV detector (λ 220 nm).

Rabbit Immunization Protocol. Immunization experiment was performed with GNP1, V3β-GNP1, and V3β-GNP3. Five female New Zealand White rabbits (approximately 2 kg) were immunized with each nanoparticle. Injections were performed intramuscularly in the inferior extremities with a total volume of 400 μL solution per animal and immunization (200 μL per injection). Prime was performed with 60 μg of nanoparticles in the presence of 10 μg of Monophosphoryl Lipid A (MPL) and 20 μg of Quil-A as adjuvants. After 34 days, rabbits were boosted with 50 μg of nanoparticles and blood was extracted after 8 and 12 days from the boost (B1−8 and B12). After 34 days, the second and last boost was performed with 50 μg of nanoparticles and blood was extracted after 8 and 12 days from the boost.

GNP-ELISA. NUNC Maxisorp ELISA plate wells were coated with 50 μL of control GNPs (20 μg/mL), 50 μL of V3β-GNPs (20 μg/mL which contain 0.001 mM of V3 peptides), or 50 μL of V3 peptide (0.001 mM). All solutions were prepared with coating buffer (50 mM Na_2CO_3, pH = 9.7). After 2 h at room temperature, solutions were removed and plate wells were washed with PBS (10 mM, pH = 7.4; 2 × 200 μL). BSA solution (1% in PBS) was placed in each well (200 μL) and left for 30 min at room temperature. BSA solution was removed and 100 μL of 447-52D Ab (0.09 μg/mL) or 100 μL of mice serum (1:100 in assay buffer: 0.5% BSA) was added and left under shaking 1 h at 500 rpm. ELISA plate wells were washed with PBS (3 × 200 μL) and 100 μL of anti-human horseradish peroxidase (0.8 μg/mL, Life Technologies, Novex Goat anti-Human IgG-HRP) or 100 μL of anti-mouse horseradish peroxidase (0.8 μg/mL, Life Technologies, Novex Rabbit anti-Mouse IgG-HRP) was added and left under shaking 1 h at 500 rpm. Wells were washed with PBS (3 × 200 μL) and 100 μL of substrate solution of 3,3′,5,5′-tetramethylbenzidine (TMB) in citric/acetate buffer (pH = 4) was added. After 2 min at room temperature the reaction was stopped with 50 μL of H_2SO_4 (0.8 M) and the absorbance was measured at 450 nm in an ELISA plate reader. These experiments were performed in triplicate.

Neutralization Assays. Viral supernatants were obtained from 293T cells transfection of pJR-Renilla plasmid.[43] Viral supernatants were previously titrated in U87-CCR5 cells and aliquoted and stored at −80 °C until use. Rabbit sera were used to treat viral supernatants at four different dilutions 1/5, 1/20, 1/80, and 1/320 for 45 min at 37 °C. Afterward, treated and nontreated supernatants were used to infect U87-CCR5 cells seeded in 96 microwell plates (20 000 cells/w) with a final dilution to achieve 100 000 RLUs per well. Cells were left in culture for 48 h at 37 °C and humidified atmosphere with 5% CO_2. The culture was lysed and Renilla luciferase activity measured with a Renilla assay system (Promega) following the manufacturing instructions in an illuminometer (Berthold Detections Systems).

■ ASSOCIATED CONTENT

Supporting Information

Experimental details and supporting results. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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