Published in final edited form as:

ACS Nano. 2016 October 25; 10(10): 9274–9286. doi:10.1021/acsnano.6b03409.

Switching the Immunogenicity of Peptide Assemblies Using Surface Properties

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Abstract

Biomaterials created from supramolecular peptides, proteins, and their derivatives have been receiving increasing interest for both immunological applications such as vaccines and immunotherapies, as well as ostensibly non-immunological applications such as therapeutic delivery or tissue engineering. However, simple rules for either maximizing immunogenicity or abolishing it have yet to be elucidated, even though immunogenicity is a prime consideration for the design of any supramolecular biomaterial intended for use *in vivo*. Here we investigated a range of physicochemical properties of fibrillized peptide biomaterials, identifying negative surface charge as a means for completely abolishing antibody and T cell responses against them in mice, even when they display a competent epitope. The work was facilitated by the modularity of the materials, which enabled the generation of a set of co-assembled fibrillar peptide materials with broad ranges of surface properties. It was found that negative surface charge, provided *via* negatively charged amino acid residues, prevented T cell and antibody responses to antigen-carrying assemblies because it prevented uptake of the materials by antigen presenting cells (APCs), which in turn prevented presentation of the epitope peptide in the APCs' major histocompatibility (MHC) class II molecules. Conversely, positive surface charge augmented the uptake of fibrillized peptides by APCs. These findings suggest that some surface characteristics such as extensive negative charge should be avoided in vaccine design using supramolecular peptide assemblies. More importantly, it provides a strategy to switch off potentially problematic immunogenicity for using these materials in non-immunological applications.

Graphical abstract

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None of the authors have financial conflicts of interest to disclose.

Supporting Information

Supporting Information is available free of charge *via* the Internet at <http://pubs.acs.org>. These materials include Table S1, a summary of synthesized peptides; and Figure S1, antibody isotype responses.

Keywords

nanofiber; supramolecular; immunogenicity; vaccine; biomaterial

Supramolecular or “self-assembling” nanomaterials have been increasingly investigated as platforms for immunological applications such as vaccines and immunotherapies.^{1–11} In parallel they have been investigated for many other traditionally non-immunological applications such as tissue engineering, cell scaffolds, wound repair, and drug delivery.^{12–22} In both contexts, it is of paramount importance to elicit appropriate immune responses, yet one set of applications demands maximal responses (vaccines, immunotherapies) while another demands attenuated responses (tissue engineering, drug delivery). Simple “switches” for adjusting immunogenicity in supramolecular nanomaterials would be extremely useful in both contexts, but at present the most reliable strategy is to either include strong T and B cell epitopes or avoid them altogether.² Unfortunately such a strategy may not be tractable in many cases. For example, it may be challenging to avoid immunogenicity if a therapeutic peptide or protein nanomaterial incidentally contains one or more immunogenic epitopes and removal of the epitope ablates the therapeutic function. Therefore, in the present work we sought to develop simple methods for adjusting immunogenicity in supramolecular peptide nanomaterials.

We focused our work on fibrillizing peptides. Among supramolecular materials, fibrillar assemblies of peptides and peptide derivatives have received particular interest in a wide range of biomedical applications, both those that require immunogenicity^{2–4} and those that may need to avoid it.^{14, 15} Our group has focused on the self-assembling peptide Q11 (QQKFQFQFEQQ), which assembles into nanofibers and hydrogels in physiological conditions. Epitope-bearing nanofibers can be formed by synthesizing Q11 peptides with various epitopes attached at their termini and allowing them to assemble, and these nanofibers are able to induce robust antigen-specific immune responses^{2–4} with minimal associated inflammation.³ Multiple peptides with different terminal epitopes can also co-assemble into the same nanofiber with stoichiometric precision,²³ which allows for the tuning of the epitope content within the nanofibers. By varying the ratio of T cell to B cell epitopes, for example, the potency and phenotype of immune responses can be adjusted.² This modularity was exploited in the present work. Previously it has been determined that oligomerization and the presence of both B cell and T cell epitopes within the peptide sequence are prerequisites for immunogenicity in supramolecular peptide biomaterials.^{1, 2, 24} However, if both these criteria are met, there is little current knowledge regarding how to otherwise tune the immunogenicity with precision.

For other nanomaterials such as polymeric nanoparticles and liposomes, considerable previous work has investigated the extent to which size, shape, and surface chemistry influence tissue distribution, cellular uptake, clearance, and cytotoxicity, primarily in the context of drug delivery.^{25–29} Other studies have investigated how such properties affect nanoparticle uptake by immune cells^{30–32} and how they influence the overall immune response.^{33–39} For instance, the DeSimone group utilized the Particle Replication in Non-Wetting Templates (PRINT) process to prepare nanoparticles with either negatively or positively charged surfaces, holding other materials parameters constant. Following pulmonary immunizations, cationic but not anionic nanoparticles were able to stimulate germinal center B cell expansion, CD4 T cell activation, and both systemic and mucosal antibody responses.³⁴ The Hubbell group reported that nanoparticle surface chemistry had a significant influence on immunogenicity because surface hydroxyl groups could activate complement.³⁵ For fibrillar peptide self-assemblies, it is not known to what extent size, shape, hydrophobicity, and surface potential affect immunogenicity, yet these previous reports suggested that some amount of control over immunogenicity could be gained by adjusting these properties. Therefore, we sought to systematically compare the relative importance of each of these properties, exploiting the modular construction of self-assembled peptide nanofibers to do so. Peptides based on the Q11 self-assembling sequence have been shown previously to be capable of assembling into regular nanofibers even when appended with a wide range of N-terminal functionalities, including variously charged, hydrophilic, and hydrophobic amino acid sequences,^{4,13,15,23,24} so we used this property in the present work to investigate a wide range of nanofiber properties. We found that among all of the modifications studied, negative surface potential had by far the most significant influence on immunogenicity, to the point where immune responses against epitope-containing Q11 nanofibers could be switched off completely. This is an important and surprising finding because it provides a simple design rule for avoiding surface characteristics that may compromise immunogenicity for vaccines based on this system. Even more significantly, it suggests a strategy for cloaking peptide assemblies that contain immunologically problematic epitopes in a way that renders them non-immunogenic if an application demands it.

RESULTS

Physiochemical characterization of supramolecular nanofibers

To systematically compare the relative importance of positive surface charge, negative surface charge, hydrophobicity, and hydrophilicity, we generated a series of Q11-based peptides having a variety of different terminal functionalities (Table 1). Expected m/z values were found by MALDI for each peptide (Table S1). N-terminal lysine or glutamic acid were employed for positive or negative terminal charges, respectively, while oligoethylene glycol (OEG) or propyl groups adjusted the relative hydrophilicity. We then created intermixed nanofibers containing both epitope-bearing peptides (OVAQ11, containing the OVA_{323–339} mixed B cell/T cell epitope peptide) and the variously functionalized peptides. We have previously demonstrated that several Q11 based peptides carrying different terminal functional sequences co-assemble into the same nanofiber, and we have developed preparation procedures for maximizing intermixing.²³ In addition, two different peptides

with N, N-dimethylated glutamine residues in the Q11 domain were also studied, where the dimethylated Gln residues were expected to disrupt β -sheet formation by disrupting side chain hydrogen bonding.⁴⁰

We first characterized the fibrillization, conformation, and zeta potentials of nanofibers assembled from the N-terminally modified peptides alone and epitope-bearing nanofibers containing 10 mol% OVAQ11, made by co-assembling the modified peptides and OVAQ11 at a 9:1 molar ratio. Positively charged KQ11 and KKKQ11 peptides formed long, uniform fibrils by Transmission Electron Microscopy (TEM) (Figure 1), with widths of 6–10 nm and lengths greater than 1000 nm (Table 2). They also formed similar fibrils when mixed with OVAQ11. By circular dichroism (CD), positively charged KQ11 and KKKQ11 exhibited β -sheet character, having broad minima between 216–224 nm and maxima near 205 nm (Figure 2a). Thioflavin T (ThT) binding was diminished compared to unmodified Q11 (Figure 2b), but mixing KQ11 and KKKQ11 9:1 with OVAQ11 restored ThT binding considerably (Figure 2c). The zeta potentials of KQ11 and KKKQ11 fibers were markedly positive, regardless of whether OVAQ11 was coassembled with it (Figure 3), with KKKQ11 conferring a greater zeta potential to the fibers than KQ11 as expected.

Negatively charged EQ11 and EEEQ11, like Q11 and the positively charged variants, formed long, uniform, and regular nanofibers by TEM (Figure 1), both by themselves and when mixed with OVAQ11. EEEQ11 by itself formed somewhat thinner fibers compared with EQ11 and Q11, with average widths around 5 nm, but this effect was eliminated when EQ11 was mixed 9:1 with OVA-Q11 (Figure 1, far right panel). By CD, EQ11 was observed to have pronounced maxima and minima, in similar positions to Q11 and the positively charged variants (Figure 2a). Conversely, EEEQ11 had a weaker CD signal. This would have suggested that EEEQ11 had attenuated β -sheet character were it not for subsequent ThT binding measurements, which indicated significant β -sheet fibrillar structure for EQ11 and for EEEQ11, both of which had stronger ThT-binding signals than unmodified Q11 (Figure 2b). When mixed 9:1 with OVAQ11, the negatively charged variants continued to exhibit significant ThT binding, the EQ11 signal being greater than Q11's and the EEEQ11 signal being slightly less than Q11's (Figure 2c). Taken together, the TEM, CD, and ThT-binding data suggested that one N-terminal Glu residue had negligible influence on the β -sheet fibrillar character of Q11, and three N-terminal Glu residues still enabled strong β -sheet fibril formation, especially for epitope-bearing nanofibers. Meaningful attenuation of assembly was only observed for EEEQ11 by itself. Nanofibers containing N-terminal Glu residues had zeta potentials that were markedly negative (Figure 3), with EEEQ11 conferring slightly more negative charge than EQ11 when co-assembled with OVAQ11 (Figure 3b). Taken together with the characterization data for the positively charged variants, it was concluded that N-terminally charged Q11 derivatives could be used to make drastic changes in the surface charge of epitope-bearing nanofibers, both positively and negatively, with negligible differences in fibrillar assembly.

Surprisingly, dimethylation of the side chains of the two central glutamines (Q11-2) or dimethylation of all of the glutamines (Q11-6) in the Q11 domain did not abolish fibrillization entirely, although nanofibers containing Q11-6 were slightly thinner than others (4.5 nm average width) and appeared to be more flexible, in that they had shorter persistence

lengths by TEM (Figure 1, Table 2). CD indicated that both Q11-2 and Q11-6 were largely unstructured (Figure 2a), indicating a major disruption of β -sheet folding by dimethylation. Similarly, ThT signals were nearly abolished for both Q11-2 and Q11-6 (Figure 2b and c). With respect to surface charge, the zeta potential of Q11-2 was similarly neutral compared to Q11, and that of Q11-6 was slightly negative (Figure 3a). These trends remained when Q11-2 and Q11-6 were combined with OVAQ11 (Figure 3b). Collectively, dimethylation of the glutamines caused increasing disruption of fibrillization with minor influences on surface charge.

Adjustment of the hydrophilicity of Q11 had a smaller effect on the folding and surface properties of formed nanofibers. OEGQ11 and propylQ11 formed nanofibers indistinguishable from Q11 (Figure 1), and both were predominantly β -sheet by CD (Figure 2a). PropylQ11 bound ThT well, both by itself or when co-assembled with OVAQ11 (Figure 2 b and c), while OEGQ11 showed diminished ThT binding (Figure 2b and c). This diminished ThT binding was likely a result of the OEG groups obscuring the ThT binding sites, as both CD and TEM indicated strong β -sheet fibrillar character. Neither propyl groups nor OEG groups significantly altered the neutral zeta potential of Q11, and this was true for both individual peptides and those mixed with epitope-bearing OVAQ11 (Figure 3a and b).

In sum, the characterization of all of the N-terminally modified Q11 derivatives indicated that N-terminal Glu and Lys residues had a strong influence on nanofiber zeta potential with negligible or small influences on fibrillar structure, OEG and propyl modifications had a negligible impact on fiber assembly and zeta potential, and N, N-dimethylation of the Gln residues significantly disrupted fibrillar assembly but had a small impact on zeta potential.

Surface potential-dependent antibody and T cell responses

Mice were immunized with Q11 or modified peptides fibrillized with OVAQ11 at a 9:1 molar ratio. Consistent with our previous reports,^{3, 4, 41} unmodified Q11/OVAQ11 nanofibers induced OVA-specific antibody responses after one boost and served as a baseline for the study (Figure 4). Among the modified peptides, those bearing negatively charged N-terminal amino acids exerted the most dramatic effect, significantly diminishing antibody responses in the case of EQ11 and abolishing them entirely in the case of EEEQ11 (Figure 4). PropylQ11, Q11-2, and Q11-6 also led to diminished antibody responses, with Q11-6 showing the most dramatic reduction among the three (Figure 4). Nanofibers with N-terminal lysine residues (KQ11 or KKKQ11) or hydrophilic OEG were equivalently immunogenic compared to Q11. For these groups, no diminishment of antibody responses was observed.

The abolishment of antibody responses by EEEQ11 was complete. No mice in two independently studied groups of 4 raised even a detectable titer. Even for Q11-6, which had severely compromised β -sheet structure, one mouse raised a titer of 3 (Figure 4). In this way, the negative charge provided by EEEQ11 was an even stronger means for preventing antibody responses than broad disruption of the nanofiber, which itself was already known as a strategy to avoid antibody responses.²⁴ Even one negative charge on the N-terminus of Q11, provided by one glutamate residue, was sufficient to significantly diminish antibody responses (Figure 4). Conversely, positively charged N-terminal residues had no influence

on the ability of Q11 nanofibers to raise antibody responses, a stark difference. Interestingly, across all formulations that were able to raise responses, antibody isotype profiles were similar, with IgG1 being the dominant isotype (Figure S1a). This result indicated that the various N-terminal modifications did not drastically influence the phenotype of the immune response, at least with respect to antibody isotypes. It is noteworthy that the immunogenicity did not correlate strongly with β -sheet content measured by CD or ThT binding. EEEQ11 and EQ11 maintained high β -sheet propensity but failed to induce strong immune responses. For Q11-6 β -sheet content was diminished but it also had a slight negative charge, so it is possible that the significant diminishment of antibody responses was a result of both factors. In sum, the analysis of antibody responses revealed the surprisingly strong influence of negative charge on the immunogenicity of Q11-based nanofibers.

From this point in the study onward, we refined our investigation to Q11, KQ11, EEEQ11 and Q11-6. Q11 and KQ11 represented nanofibers with undiminished immunogenicity, while EEEQ11 and Q11-6 represented peptides with significant diminishment. These groups were studied with respect to CD4 T cell responses, lymphatic draining, and uptake and presentation by antigen presenting cells. We first investigated whether the results observed for antibody responses remained consistent for CD4 T cell responses, using ELISPOT analysis (Figure 5). After 12 weeks of the immunization regimen (see methods), mice were boosted one more time and draining lymph nodes (inguinal, axillary, and brachial) were collected. Cells from the lymph nodes were cultured and stimulated with 5 μ M OVA peptide, and cells secreting IFN- γ or IL-4 were quantified. By this measure, Q11 and KQ11 groups had strong CD4 T cell responses, while EEEQ11 and Q11-6 groups failed to induce any significant T cell responses (Figure 5). These results were quite consistent with the antibody studies: Both EEEQ11 and Q11-6 were incapable of raising T cell responses. The Q11-6 group had one weakly responding mouse, but there was no detectable T cell response at all for EEEQ11 (Figure 5, inset).

Efficient lymphatic draining of modified nanofibers

Having found that nanofiber zeta potential contributes strongly to the overall immunogenicity, we tested the hypothesis that surface properties may influence lymphatic draining and trafficking of the nanofibers. Poor entry into lymph nodes could result in compromised immune responses, as lymphatic draining is critical for many nano-engineered immunotherapies.^{1, 42–44} Peptide nanofibers containing modified peptides, 10 mol% OVAQ11, and 1 mol% fluorescent TAMRA-OVAQ11 were prepared to track the lymphatic draining using *ex vivo* imaging. Draining lymph nodes (brachial, axillary, and inguinal) were collected and imaged using an IVIS 200 imager 24 h after subcutaneous immunization. This time point was chosen to emphasize draining of the assemblies rather than slower trafficking of the materials by antigen-presenting cells. By this measure, all peptide nanofibers were drained to the lymph nodes (Figure 6). Interestingly, Q11-6 drained most efficiently, with considerably more accumulation in all lymph nodes tested than Q11. It is not clear why this was the case, but it is known that size is an important determinant of whether a particle can enter the lymphatic system and drain efficiently to lymph nodes, and that particles smaller than 200 nm drain with particular efficiency.⁴⁴ It is not clear to what extent such metrics would apply to the high aspect ratio nanofibers studied here, but it is possible that Q11-6's

increased lymph node drainage could have arisen from smaller size (widths of about $\frac{1}{2}$ that of other fibers, Table 2) and potential degradation into smaller particles. KQ11 and EEEQ11 trafficked similarly to each other (Figure 6), at a level slightly greater than Q11 (Figure 6). These findings indicated that surface charge did not significantly affect gross lymphatic draining, and that the diminished responses seen with the negatively charged assemblies could not be attributed to reduced entry into lymph nodes.

Differential uptake and presentation efficiency by antigen presenting cells

For the activation of antigen-specific T helper cells, antigens need to be internalized by antigen-presenting cells (APCs), proteolyzed (processed) into short T cell epitopes, loaded into class-II major histocompatibility molecules (MHC class II), and presented to T cells on the APC surface. Antigen-specific T cells, once activated, then go on to help B cells produce antibodies. Owing to the fact that both T cell responses and B cell responses were eliminated by negatively charged EEEQ11, we hypothesized that significant charge on the nanofibers may interfere with the process of antigen uptake, processing, and presentation. To test this hypothesis, we studied nanofiber internalization using fluorescent nanofibers and flow cytometry, followed by subsequent experiments with reporter cells (DOBW) that can detect appropriately processed and MHC class II-presented epitopes in cultures of APCs. TAMRA-labeled nanofibers were injected intraperitoneally, and cells in intraperitoneal lavage fluid were collected and stained for flow cytometry. Dendritic cells were gated as the F4/80-negative/MHC class II-high CD11c+ population, while macrophages were gated as the F4/80-positive population. For the Q11 group, 18.1% of dendritic cells and 20.9% of macrophages were found to be TAMRA-positive; that is, these cells had acquired peptide nanofibers (Figure 7). In stark contrast, for EEEQ11 and Q11-6, negligible uptake by macrophages or DCs was observed (Figure 7). For dendritic cells, this effect was especially pronounced (Figure 7b, inset). Interestingly, the uptake of positively charged KQ11 containing nanofibers was considerably greater than even Q11, to the point that around half of DCs and almost all macrophages took up KQ11 nanofibers (Figure 7b). To control the different doses of nanofibers received by APCs *in vivo*, the efficiency of cell internalization was further studied *in vitro* using cultured bone marrow dendritic cells (BMDCs). Exact doses of fluorescently labeled nanofibers with different surface charges were added to CpG-stimulated BMDCs, and nanofiber uptake was evaluated by flow cytometry. Results from this *in vitro* experiment corresponded strikingly with observations from intraperitoneally delivered nanofibers (Figure 7c). In both cases, positive charge augmented nanofiber uptake by dendritic cells and macrophages, and negative charge abolished it. The vast majority of Flt-3L stimulated cells were dendritic cells (>97%), but macrophages were a detectable component of these cultures (less than 3%), so uptake by both populations is reported in Figure 7c. Overall these findings correspond with the previous findings of DeSimone and colleagues, who observed that positive charge enhanced nanoparticle uptake and subsequent immune responses for PRINT nanoparticles.^{33, 34}

Since the uptake study by flow cytometry could not differentiate whether fluorescently labeled cells had actually internalized the peptides rather than merely binding them on their surfaces, presentation efficiency was investigated further. DOBW cells are hybridomas that produce IL-2 when they encounter APCs presenting the OVA epitope in MHC class II

(Figure 8a). Therefore, the concentration of IL-2 from the culture medium is an accurate indicator of presentation efficiency. Mice were intraperitoneally immunized with nanofibers containing OVAQ11 and the variously modified Q11 peptides, and cells from intraperitoneal lavage fluid were collected 18 h later and cultured *ex vivo* with DOBW cells for 20 h. Concentrations of IL-2 in cell culture medium were quantified by ELISA. By themselves, without co-culturing with DOBW cells, cells from lavage fluid did not produce any IL-2 (Figure 8b). Coinciding with the flow cytometry results described above, Q11-based nanofibers were processed and presented, as reflected by significant IL-2 production by DOBW cells (Figure 8c). KQ11-based nanofibers showed an even higher presentation efficiency (Figure 8c), consistent with the uptake experiments. For EEEQ11 and Q11-6, neither was capable of being processed to stimulate DOBW T cells, as neither group produced any IL-2 above background (Figure 8c). That is, APCs failed to present OVA in their surface MHC class II when the antigen was supplied to the APCs in the form of nanofibers containing either EEEQ11 or Q11-6. These findings, along with the flow cytometry experiments described above (Figure 7), demonstrated that surface potential could be employed to diminish the ability of APCs to acquire and present nanofiber-associated antigens (in the case of negative charge) or to enhance nanofiber uptake (in the case of positive charge).

Titration surface potential in nanofiber vaccines

Having established that the adjustment of surface charge could be used to eliminate the immunogenicity of self-assembled peptide nanofibers, we then investigated whether nanofibers could contain a lesser amount of charged peptides and still maintain their immunogenicity. N-terminally modified peptides (0.67 mM) were intermixed with OVAQ11 (1.33 mM) at a 1:2 molar ratio. These nanofibers contained 33 mol % modified peptide and 67 mol% OVAQ11, considerably less modified peptide than the 90 mol% present in the previously studied nanofibers reported above. The new nanofibers had similar morphology and dimensions (Figure 9a), and all had a slightly positive charge (Figure 9b). All peptide nanofibers also bound ThT, indicating significant β -sheet content (Figure 9c). In subcutaneous immunizations, all peptide nanofibers induced comparable OVA specific IgG titers (Figure 9d) over 12 weeks with similar IgG isotype profiles (Figure S1b). This study suggested that in order for negative surface potentials to diminish the immunogenicity of peptide nanofibers, a threshold amount of surface charge must be provided. In the absence of significant adjustments to nanofiber zeta potential, equivalent immune responses can be expected.

Negative charges did not introduce immune tolerance

To further elucidate how negative charges could modulate immune responses, our final experiment in this study investigated whether immune tolerance was introduced by negative charges. In one group, mice were immunized three times with negatively charged OVA-containing nanofibers and then immunized with neutrally charged OVA-containing nanofibers, to determine if the negatively charged formulations induced some form of inhibitory effect. Strikingly, the neutrally charged material was rapidly immunogenic, even after multiple previous immunizations with negatively charged nanofibers, indicating that tolerance had not been brought about (Figure 10, Group 1). In a second group, mice were

first immunized with neutrally charged OVA-containing nanofibers and then with negatively charged OVA-containing nanofibers. Again, no tolerizing effect was observed (Figure 10, Group 2). Hence, negatively charged nanofibers could neither prevent the immunogenicity of neutrally charged materials nor induce tolerance for established immune responses.

DISCUSSION

It is known that surface properties can significantly affect the fate and efficacy of nanomaterials *in vivo* and *in vitro*,^{25, 26} and with respect to immunogenicity, nanomaterial properties have been adjusted previously to steer the immune response towards an antigenic payload.^{34, 42, 43} However, the extent to which these principles may apply to peptide assemblies or other supramolecular materials has remained to be elucidated, especially with regards to overall immune responses. The importance of the present work is that it illustrates a simple design rule, the use of negative charge, to limit the immunogenicity of self-assembled peptide nanofibers. This effect was surprisingly strong, as all measured immune responses against negatively charged peptide nanofibers were not merely attenuated, but abolished. In contrast, positively charged nanofibers were equivalently immunogenic compared to neutrally charged nanofibers, and their uptake by antigen presenting cells was enhanced considerably (results summarized in Table 3).

These findings raise important considerations for a wide range of applications. For example, peptide and protein self-assemblies are under considerable investigation for many non-immunological applications such as tissue engineering and cell scaffolds,^{14, 15, 45} drug delivery,^{16, 20, 2146–48} antimicrobials,⁴⁹ wound healing,^{19, 50} and others.^{51, 52} In this broad range of applications, any antibody response against proteins, peptides, or other antigenic components incorporated into the materials could lead to blockage of the factors' function, early clearance of the material, or at worst, inflammatory rejection responses, although in general peptide assemblies have been found to be non-inflammatory to date.³ Co-assembling negatively charged peptides into supramolecular peptide materials intended for these purposes may represent a simple but powerful way of minimizing the occurrence of such sequelae. In the case of supramolecular assemblies intentionally designed to be immunogenic, such as those being investigated as vaccines and other immunotherapies by our laboratory^{2–5} and others,^{7–10} the present study suggests that significant negative charge could compromise immunogenicity and should be avoided. If, for example, a chosen epitope or antigen in a supramolecular vaccine formulation happens to carry a significant negative charge, it may be judicious to either counter the negative charge with other positively charged molecules in the nanomaterial or re-engineer the epitope.

In the present study we did not explore in mechanistic detail the specific reason why negatively charged peptide self-assemblies were not acquired, processed, and presented by antigen presenting cells, but one can speculate why this occurred. The simplest explanation would be that excess negative charge led to repulsion between the negatively charged surface of antigen-presenting cells and the self-assemblies. Positive charge on nanoparticles has been recently shown to enhance such processes.^{33, 34} This notion is supported by the observation that no immune tolerizing effect was established by negative charges. Also, it should be noted that the antigen acquisition and activation studies were performed for

intraperitoneal injections. It may be possible that nanofibers delivered to APCs in lymph nodes or *via* other routes may be differently acquired, but the abolishment of immune responses even for subcutaneous injections makes this unlikely.

Finally, a determination of whether this phenomenon applies to other nanomaterials would be an important future study. If nanoparticles, nanofibers, and other supramolecular nanomaterials can be rendered non-immunogenic even when they contain competent antigens, B cell epitopes, and T cell epitopes, it would be a boon for their use in biomedical applications where immunogenicity is counterproductive. As a specific example, self-assembled nanomaterials presenting targeting moieties such as proteins or antibodies would circulate for longer periods if immune responses against them were attenuated. This would be especially important for nanomaterials delivered repeatedly and thus at risk for developing immune responses against them over time. It would also be important to determine how strong the effect of negative charge is on the immunogenicity of other widely used synthetic vaccine platforms, including micelles,^{7,8} self-assembling polypeptide nanoparticles,¹⁰ polymer nanoparticles,³⁵ and other particulate-based materials. The fact that similar charge effects have been observed by others, for example in polymer nanoparticles generated by PRINT processing,^{33,34} indicates that this principle may apply well beyond self-assembled peptides, but future systematic evaluation of each of these platforms would be necessary to confirm this.

CONCLUSIONS

We investigated the immunogenicity of antigen-bearing self-assembling peptides co-assembled into nanofibers with other peptides bearing different charges, hydrophobic groups, or hydrophilic groups. We compared immune responses to these materials with those having significant attenuation of their assembly *via* N, N-dimethylation of their glutamine residues. Characterization studies revealed that charged residues placed N-terminally to the Q11 self-assembling domain had a small or negligible impact on nanofiber formation, yet immune responses were significantly modulated. Negatively charged nanofibers were incapable of raising B cell/antibody responses or T cell responses, and they were unable to be acquired and presented by antigen presenting cells. Conversely, positively charged nanofibers maintained their ability to raise T cell and B cell responses, and were acquired avidly by antigen presenting cells. These findings illustrate a simple design rule for modulating the immunogenicity of self-assembled peptide biomaterials.

METHODS

Peptide synthesis and formulation

The peptides OVAQ11 (or OVA₃₂₃₋₃₃₉-SGSG-Q11, H₂N-ISQAVHAAHAEINEAGR-SGSG-QQKFQFQFEQQ-Am), Q11 (Ac-QQKFQFQFEQQ-Am), KQ11 (Ac-K-SGSG-QQKFQFQFEQQ-Am), KKKQ11 (Ac-KKK-SGSG-QQKFQFQFEQQ-Am), EQ11 (Ac-E-SGSG-QQKFQFQFEQQ-Am), EEEQ11 (Ac-EEE-SGSG-QQKFQFQFEQQ-Am), OEGQ11 (HO(CH₂CH₂O)₂CH₂CH₂CO-QQKFQFQFEQQ-Am), and PropylQ11 (CH₃CH₂CH₂CO-SGSG-QQKFQFQFEQQ-Am) were synthesized using standard Fmoc solid-phase synthesis as previously reported.¹⁵ To prepare the two peptides with N, N-

dimethylated Gln residues (Q*), Q11-2 (Ac-QQKFQ*FQ*FEQQ-Am) and Q11-6 (Ac-Q*Q*KFQ*FQ*FEQ*Q*-Am), Fmoc-Glu(O-2-phenylisopropyl)-OH was used. Following standard Fmoc solid-phase synthesis, 2- phenylisopropyl groups were cleaved with 1% TFA in dichloromethane (DCM) for 1–2 h. Peptides were then washed with dimethylformamide (DMF) and dried under vacuum. To perform amidation, 1 mmol of benzotriazole-1-yl-oxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP) was dissolved in 5 mL DMF and mixed with 2 mL of 2 M dimethylamine in methanol.⁴⁰ This was applied to the resin and reacted overnight under nitrogen. The resin was then washed with DMF again and dried under vacuum, after which peptides were cleaved as using a standard TFA cleavage cocktail.¹⁵ OVA-Q11 was N-terminally conjugated with 5-(and-6)-carboxytetramethylrhodamine (TAMRA) using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) coupling. All peptides were purified by reverse-phase HPLC, and lyophilized as previously reported.¹⁵ Peptide identity was confirmed using matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) on a Bruker Ultraflex extreme MALDI-TOF mass spectrometer using α -cyano-4-hydroxycinnamic acid as the matrix.

To prepare immunization formulations, lyophilized peptides were weighed and intermixed by vortexing in a closed tube for 30 min. Sterile ultrapure water was added to prepare 8 mM solutions. After overnight incubation at 4 °C, peptide solutions were diluted to 2 mM in 1× PBS using sterile water and sterile 10× PBS (Fisher BP399-500) and incubated at room temperature for 3 h. These preparations were used for immunizations. For quality control, endotoxin measurements were conducted on the same peptide solutions that were used for immunizations, using the Limulus Amebocyte Lysate chromogenic endpoint assay (Lonza). Endotoxin levels of all immunization preparations were <0.5 EU/mL (<0.05 EU per 100 μ L dose).

Electron Microscopy

Peptide nanofibers prepared at 2 mM were diluted to 0.2 mM with 1× PBS and vigorously vortexed, then 5 μ L of diluted peptide nanofibers were deposited onto Formvar/Carbon coated 400 mesh copper grids (Electron Microscopy Sciences). The sample was allowed to incubate for 1 minute, then washed 5 times with 0.2 μ m filtered ultrapure water. Negative staining was performed for 1 minute with 1% w/v uranyl acetate in water. Staining solution was withdrawn with filter paper. Samples were imaged on a FEI Tecnai G2 Spirit TEM. Widths were measured in ImageJ. Measurements were made for the bright central portion of the nanofibers, and reported widths exclude the dark edges stained by uranyl acetate.

Secondary structure analysis by Circular Dichroism (CD) and Thioflavin T (ThT) binding

Prepared aqueous solutions of 2 mM peptide were diluted to 0.2 mM with 1× PBS and vigorously vortexed. Far UV CD spectra were collected on a Jasco J-1500 CD spectrometer from 260 nm to 180 nm in a 0.1 cm path length cuvette. Five accumulations were averaged for each measurement with a wavelength increment of 1 nm and scanning speed of 100 nm per minute. For ThT binding assay, 20 μ L of prepared 2mM peptide nanofibers was added to 80 μ L 0.0025 mM ThT in PBS in a 96 well plate. Fluorescence emission was scanned from 400–600 nm at a fixed excitation of 442 nm on a Tecan Infinite 200 Pro.

Zeta potential measurements

Prepared 2 mM aqueous peptides were diluted to 0.2 mM with 1× PBS and vigorously vortexed. Zeta potential was measured with a Starna DTS1070 folded capillary cell on a Malvern Nano Zeta Sizer. Samples were allowed to equilibrate at 25 °C for 3 min, and triplicate measurements were averaged.

Mice and immunizations

Female C57BL/6 mice were purchased from Harlan Sprague Dawley and housed in a centralized animal facility at the University of Chicago. All procedures were approved by the University of Chicago Institutional Animal Care and Use Committee and were in compliance with the NIH Guide for the Care and Use of Laboratory Animals. Mice were anesthetized and the preparations described above were delivered subcutaneously (50 μ L in two locations on the left and right flank). This amounts to a 20 nmol (35 μ g) dose of OVA_{323–339} epitope peptide. Booster immunizations were given after 4 weeks at the same dose of the primary immunizations. Subcutaneous injections were used to study antibody responses, T cell responses, and lymphatic draining. Intraperitoneal injections were performed to study uptake by antigen presenting cells and presentation efficiencies. These constituted two 50 μ L intraperitoneal injections in the left and right side of the abdomen.

Measurements of antibody and T cell responses

Serum collected biweekly *via* the submandibular vein was analyzed for antigen-specific IgG by ELISA. Plates were first coated with 2 μ g/mL streptavidin solution overnight at 4 °C, washed with 0.05% (w/v) tween-20 in PBS (1× PBST), blocked by Superblock blocking buffer solution (Thermo Fisher, Cat#37515), washed again, and then coated with 20 μ g/mL of biotinylated peptide OVA_{323–339} (Biotin-pOVA) for 1 h at room temperature. Serially diluted sera in 1% (w/v) BSA in 1× PBST were added to the plate, and OVA-specific IgG was detected by horseradish peroxidase conjugated Fc γ fragment specific goat anti-mouse IgG (Jackson Immuno Research, Cat #115-035-071).

To analyze T cell activation by ELISPOT, mice were euthanized 7 days after the final booster immunization. Draining lymph nodes (inguinal, brachial, and axillary) were collected, and single-cell suspensions were prepared as previously reported.^{2, 41} Briefly, 96-well ELISPOT plates (Millipore, Cat#MSIPS4510) were washed and coated overnight at 4 °C with anti-IFN- γ (BD, Cat#51-2525KZ) or anti-IL-4 (BD, Cat#51-1819KZ) capture antibody. After blocking with complete RPMI medium for 2 h at 37 °C, 200 μ L of 2.5M/mL cells were seeded and stimulated with 5 μ M pOVA for 48 h in a CO₂ incubator at 37 °C. Plates were then washed and incubated with biotinylated anti-IFN- γ (BD, Cat#51-1818KA) or anti-IL-4 (BD, Cat#51-1804KZ) detection antibodies, then with streptavidin-alkaline phosphatase (Mabtech, Cat#3310-10), and finally with the substrate Sigmafast BCIP/NBT (Sigma, Cat#B5655). Plates were allowed to dry overnight before the spots were imaged and counted using an ELISPOT reader (Cellular Technology Ltd).

Lymph node draining

Mice were immunized with fluorescent nanofibers containing 1 mol% TAMRA-OVAQ11. Draining lymph nodes were collected 24 h later and imaged using a Xenogen IVIS 200

imaging system (535 nm excitation, 600 nm emission). Images were processed and quantified by the IVIS 200 software.

Cell uptake and presentation

Mice were euthanized 18 h after intraperitoneal (IP) immunization of fluorescently labeled nanofibers containing 1 mol% TAMRA-OVAQ11. IP lavage was conducted using an intraperitoneal injection of 2 mL Hank's balanced salt solution (HBSS). The abdomen was massaged 40 times and about 1.2–1.5 mL of lavage fluid was withdrawn from the IP space. Cells were collected by centrifugation and washed once by flow buffer (PBS containing 2% fetal bovine serum). After blocking the Fc receptor with 2.4G2 antibody (BD Bioscience, Cat#: 553142), cells were stained for F4/80 (Biolegend, Cat#: 123128), MHC class II (Biolegend, Cat#: 107606), and CD11c (Biolegend, Cat#: 117318). Cells were washed and resuspended in flow buffer with 1 µg/mL DAPI. Flow cytometry was performed on a BD LSRII and analyzed using FlowJo software. Macrophages were gated as F4/80-positive and dendritic cells (DC) were gated as F4/80-negative/ MHC class II-high/ CD11c-positive. The percentage of TAMRA-positive cells (having internalized labeled nanofibers) was calculated for both macrophage and DC populations.

To control the doses of nanofibers exposed to APCs, cellular uptake was also studied *in vitro* using bone marrow derived dendritic cells (BMDCs). Bone (femur and tibia) marrow cells were cultured in complete RPMI medium containing 200 ng/µL Flt-3L. Loosely bound cells and floating cells were collected on day 8 for assays. Fluorescently labeled peptide nanofibers (1.8 mM peptide + 0.2 mM OVAQ11 + 0.02 mM TAMRA-OVAQ11) were diluted to 0.2 mM in complete RPMI medium containing 200 ng/mL CpG (ODN 1826). 250 µL of these diluted peptide nanofibers were added to 250 µL of cell suspension (1×10^6 cells /mL final cell concentration) and cultured for 18 h. Cells were stained for F4/80, CD11c, and MHCII for flow cytometry, as in *in vivo* studies. Again, DCs were gated as F4/80-negative/MHC class II-high/CD11c-positive. Less than 3% of cells exhibited a macrophage phenotype (F4/80-positive), and the uptake efficiency in the macrophage population was also quantified.

To investigate presentation efficiency, cells from lavage fluid were washed, resuspended at 0.5 M/mL in complete RPMI medium, and seeded into 96 well plates (100 µL per well). Next DOBW reporter T cell hybridomas were added (100 µL of 0.5 M/mL cells in complete DMEM). DOBW cells produce IL-2 when they encounter their specific epitope (OVA_{323–339}) complexed within the appropriate MHC class II (I-A^b). Co-cultures were incubated overnight in a CO₂ incubator at 37 °C. Cell culture medium was collected after a brief centrifugation (1500 rpm for 5 min), and the concentration of IL-2 in the media was measured by ELISA (BD, Cat#55148).

Statistical analysis

Statistical analysis was performed in Graphpad Prism using 1 way or 2 way ANOVA with Dunnett's multiple comparison test as indicated in figure legends. Means ± standard deviations are presented unless otherwise noted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank T. Sun for assistance with blood draws, Y. Wu for assistance with dimethylation of glutamine, C. Harding for providing the DOBW cells, and A. Chong for helpful discussions. The work described in this article was conducted at the University of Chicago and supported by the National Institutes of Health (NIBIB, 1R01EB009701; NIAID 1R01AI118182). Its contents are solely the responsibility of the authors and do not necessarily represent the official views of these agencies.

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Figure 1. Negative stained TEM images of self-assembled nanofibers

Nanofibers assembled from 2mM total peptide were diluted to 0.2 mM for spotting onto TEM grids (scale bar: 200 nm). Shown are nanofibers from each type of peptide alone (top panel for each type), plus those containing 90 mol% modified peptide and 10 mol% OVAQ11 (bottom panel for each type, produced by mixing 1.8 mM modified peptide with 0.2 mM OVAQ11, a 9:1 ratio).

Figure 2. Secondary structure characterization of modified peptides

CD spectra (a) and ThT fluorescence spectra (b) of individual modified peptides (a, b) and modified peptides mixed 9:1 with OVAQ11 (c). N, N-dimethylation of glutamine side chains particularly disrupted β -sheet structure (a–c).

Figure 3. Zeta potentials of peptide nanofibers

Lysine-terminated KQ11 and KKKQ11 produced nanofibers with positive zeta potentials, whereas glutamate-terminated EQ11 and EEEQ11 rendered nanofibers with negative zeta potentials (a, b). Shown are zeta potentials of nanofibers containing one type of peptide (a) and those formed by mixing modified peptides 9:1 with OVAQ11 such that they contained 10 mol % OVAQ11 (b). Dimethylation of all glutamine side chains (Q11-6) led to slightly negative surface potentials (a, b), whereas other modifications did not significantly alter zeta potential.

Figure 4. Antibody responses raised by nanofibers in mice

Nanofibers containing negatively charged Q11 variants showed significantly diminished (for EQ11) or abolished (for EEEQ11) titers. Mice were immunized subcutaneously with 100 μ L of 2 mM nanofibers containing 90 mol% of the modified peptides as indicated and 10 mol% OVAQ11. This amounts to a dose of 20 nmol (35 μ g) of OVA epitope peptide. Mice were boosted once at week 4 with the same formulations. Serum total IgG titers were measured by ELISA. Data were combined from two independent experiments (n=8 total) and analyzed by one-way ANOVA and Dunnett's multiple comparison test (compared to Q11/OVAQ11 group at respective time points). *:p<0.05, **: p<0.01, ***: p<0.001.

Figure 5. ELISPOT analysis of T cell responses

Similar to antibody titers, T cell responses were completely abolished against EEEQ11-containing nanofibers. Mice were immunized subcutaneously with 100 μ L 2 mM nanofibers containing 90 mol% of the modified peptides as indicated and 10 mol% OVAQ11 (20 nmol/35 μ g OVA epitope) and then boosted at week 4 and week 12 with the same formulations. OVA-specific T cell responses were measured by ELISPOT at week 13. IFN- γ or IL-4 secreting cells were quantified. The inset shows a magnified y-axis. Data were analyzed by two-way ANOVA and Dunnett's multiple comparison test (compared to naive control group). ***: $p < 0.001$.

Figure 6. Surface potentials did not compromise lymphatic draining

Draining lymph nodes were collected at 24 h after immunization, imaged (a), and quantified (b). Mice were immunized subcutaneously with 100 μ L TAMRA-labeled nanofibers (1.8 mM peptide + 0.2 mM OVAQ11 + 0.02 mM TAMRA-OVAQ11). Images shown were from one representative experiment (n=2) and data plotted were combined from two experiments (n=4) and analyzed by two-way ANOVA and Dunnett's multiple comparison test (compared to Q11 group). *:p<0.05, ***: p<0.001.

Figure 7. Nanofiber uptake by macrophages and dendritic cells was significantly modulated by surface potential, both *in vivo* and *in vitro*

Nanofibers with negative surface charge (containing EEEQ11 or Q11-6) had negligible uptake while nanofibers with positive surface charge (KQ11) were internalized to a greater degree than Q11, both *in vivo* (a, b) and *in vitro* (c). Representative flow cytometry data for intraperitoneal injections are shown in (a) and quantified in (b), inset shows magnified y-axis. Quantified uptake data for *in vitro* cultures of macrophages and BMDCs are shown in (c). For intraperitoneal injections, mice received 100 μ L TAMRA-labeled nanofibers (1.8 mM peptide + 0.2 mM OVAQ11 + 0.02 mM TAMRA-OVAQ11; corresponding to an epitope dose of 20 nmol/35 μ g OVA epitope). Dendritic cells were defined as F4/80[−]/MHC class II high/CD11c⁺, macrophages were F4/80⁺, and the TAMRA signal was used to identify cells that had taken up the nanofibers. For *in vitro* delivery, Flt-3L-stimulated bone marrow-derived cells were cultured with 0.1 mM fluorescent peptide nanofibers. The same flow gating as above was used to distinguish DCs (over 97% of the cells) from a minor fraction of macrophages, and to quantify the percentage of each population that took up the TAMRA-labeled nanofibers. Data shown were combined from two independent experiments (b, n=6; n=3 for naive control) or from one independent experiment (c, n=4). Data were analyzed by

one-way ANOVA and Dunnett's multiple comparison test (compared to naive control group). ***: $p < 0.001$.

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Figure 8. Antigen presentation depended strongly on nanofiber surface potential

Presentation of the OVA epitope in MHC class II molecules was measured using DOBW reporter T cells. Mice were immunized intraperitoneally with 100 μ L 2 mM nanofibers containing 90 mol% of the modified peptides as indicated and 10 mol% OVAQ11 (20 nmol/35 μ g OVA epitope dose), and APCs were recovered *via* IP lavage. These cells were co-cultured with DOBW cells, which secrete IL-2 upon encountering cellsurface MHC class II loaded with the OVA epitope (a). By themselves, cells from the IP lavage did not produce IL-2 (b). Negatively charged nanofibers (containing EEEQ11 or Q11-6) exhibited negligible presentation, whereas nanofibers containing positive KQ11 were presented more efficiently than Q11 (c, inset shows magnified y-axis). Data shown were from one representative experiment (n=4) and analyzed by one way ANOVA and Dunnett's multiple comparison test (compared to naive control group). *:p<0.05, ***: p<0.001.

Figure 9. Nanofibers containing lower quantities of modified peptides retained more uniform zeta potentials and immunogenicity

Q11, KQ11, EQ11, OEGQ11, or PropylQ11 were co-assembled with OVAQ11 to produce nanofibers containing 33 mol% modified peptide and 67 mol% OVAQ11. All formed nanofibers (a), had slightly positive surface potentials (b), and had dominant β -sheet structure (c). Mice were immunized subcutaneously with 100 μ L 2 mM nanofibers (134 nmol/238 μ g OVA epitope dose). Mice were boosted at week 4. All nanofibers raised strong antibody responses (d). Data were combined from two independent experiments ($n=9$ total) and analyzed by one-way ANOVA and Dunnett's multiple comparison test (compared to Q11/OVAQ11 group). No statistical differences were found.

Figure 10. Negatively charged nanofibers could not develop immune tolerance against subsequently delivered neutrally charged nanofibers, nor could they tolerize established immune responses

Mice were immunized subcutaneously with either neutral (Q11) or negatively charged (EEEQ11) nanofibers containing OVA and boosted at weeks 4, 8, and 10 with the formulations indicated in the figure (90 mol% modified peptides and 10 mol% OVAQ11, 2 mM total peptide, 100 μ L total volume, amounting to 20 nmol (35 μ g) of OVA epitope peptide). Repeated administration of EEEQ11 did not prevent subsequent immunogenicity of Q11 (Group 1), and immune responses established with Q11 were not tolerized by subsequent EEEQ11 administration (Group 2). Data shown were from one independent

experiment (n=5) and analyzed by one-way ANOVA and Dunnett's multiple comparison test (compared to Q11 group). **: $p < 0.01$, ns: not significant.

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Table 1

Summary of peptides investigated

Peptide	Sequence	Property
Q11	QQKFQFQFEQQ	Base material
KQ11	K-SGSG-Q11	Positive charge
KKKQ11	KKK-SGSG-Q11	Positive charge
EQ11	E-SGSG-Q11	Negative charge
EEEQ11	EEE-SGSG-Q11	Negative charge
OEGQ11	HO(CH ₂ CH ₂ O) ₂ CH ₂ CH ₂ C(O)-Q11	Hydrophilicity
PropylQ11	CH ₃ CH ₂ CH ₂ C(O)-SGSG-Q11	Hydrophobicity
Q11-2	QQKFQ*FQ*FEQQ	Structure breaking
Q11-6	Q*Q*KFQ*FQ*FEQ*Q*	Structure breaking

Q*: N, N-dimethyl Glutamine

Table 2

Quantified diameters (mean $\pm$ S.D.) of nanofibers from TEM images.

	Q11	KQ11	KKKQ11	EQ11	EEEQ11	OEGQ11	PropylQ11	Q11-2	Q11-6
Peptide	9.18 $\pm$ 1.67	8.51 $\pm$ 1.12	9.66 $\pm$ 0.99	11.56 $\pm$ 1.58	7.98 $\pm$ 1.98	9.51 $\pm$ 1.06	11.84 $\pm$ 1.84	10.42 $\pm$ 1.21	4.54 $\pm$ 0.96
9:1 with OVAQ11	11.19 $\pm$ 1.67	10.47 $\pm$ 1.77	10.47 $\pm$ 1.82	12.28 $\pm$ 2.02	12.46 $\pm$ 2.08	10.76 $\pm$ 0.91	10.85 $\pm$ 1.55	10.33 $\pm$ 2.14	4.71 $\pm$ 0.81

Table 3

Summary of properties and immunogenicity of vaccine formulations

Formulation (9:1)	Nanofiber	Conformation	Zeta potential	Antibody response	Lymphatic draining	DC uptake presentation	T cell activation
Q11/OVAQ11	Yes	β -sheet	-0.1 mV	Yes	Yes	Yes	Yes
KQ11/OVAQ11	Yes	β -sheet	+16.0 mV	Yes	Yes	Yes	Yes
EEEQ11/OVAQ11	Yes	β -sheet	-25.2 mV	No	Yes	No	No
Q11-6/OVAQ11	Yes	random coil	-10.6 mV	No	Yes	No	No