Ovalbumin self-assembles into amyloid nanosheets that elicit immune responses and facilitate sustained drug release

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Abstract

Amyloid fibrils are associated with many neurodegenerative diseases, motivating investigations into their structure and function. Although not linked to a specific disease, albumins have been reported to form many structural aggregates. We were interested in investigating host immune responses to amyloid fibrils assembled from the model protein ovalbumin. Surprisingly, upon subjecting ovalbumin to standard denaturing conditions, we encountered giant protein nanosheets harbouring amyloid-like features and hypothesized that these nanosheets might have potential in clinical or therapeutic applications. We found that the nanosheets, without the administration of any additional adjuvant, evoked a strong antibody response in mice that was higher than that observed for native ovalbumin. This suggests that amyloid nanosheets have self-adjuvanting property. The nanosheet-induced immune response was helper T cell 2 (Th2) biased and negligibly inflammatory. While testing whether the nanosheets might form depots for the sustained release of precursor proteins, we did observe release of ovalbumin that mimicked the conformation of native protein. Moreover, the nanosheets could load the anticancer drug doxorubicin and release it in a slow and sustained manner. Taken together, our results suggest that amyloid nanosheets should be further investigated as either an antigen delivery vehicle or as a multifunctional antigen and drug co-delivery system, with potential applications in simultaneous immunotherapy and chemotherapy.

Self-assembly of proteins into amyloid fibrils, the highly ordered nanostructures harboring cross β-cores, has been associated with various neurodegenerative disorders (1-3). On the contrary, amyloids have been reported to
perform normal biological functions including hormone storage and memory persistence (4,5). Owing to high thermal stability, stiffness, biocompatibility, and controllable self-assembly (6,7); amyloids are emerging as novel class of biological nanomaterials. They have found applications in the areas of food science, materials science, medicine and electronics (8-12). Amyloid fibrils have also been hybridized with graphene and DNA origami to develop hybrid biomaterials (7, 13-15). Moreover, amyloid based supramolecular assemblies are gaining attention as immune adjuvant and antigen delivery systems because of their potential to release the precursor protein/peptide and resistance to proteolytic attack that extends their residence time in circulation (16). This feature allows them to boost the immune system time and again in the absence of external boosters.

Under specific denaturation conditions, ovalbumin has been reported to form aggregates in vitro (8). A decrease in α-helical content, binding of thioflavin T, or Congo red to the albumin aggregates suggest formation of β-sheet type of structures (17). Albumin aggregates of diverse morphologies have been reported and interestingly, the morphologies obtained seem to have direct correlation with the kind of denaturing environment provided to the protein. For example, human serum albumin (the human counterpart of ovalbumin) has been observed to form both fibrillar and non-fibrillar aggregates under various environmental conditions (17). Like fibrillar aggregates, some of its non-fibrillar aggregates (displaying a certain filamentous structure but not the conventional fibril form) have been found to possess a typical negative peak in CD spectra at wavelengths close to 220 nm and bind Congo red. The different precursor states that result from different solution conditions can generate fibrillar or non-fibrillar aggregates with similar β-sheet content but with distinct morphological features. For human serum albumin, flat ribbons observed are very much different from cylindrical forms of amyloid fibrils and relatively thick rodlike specimens, although, each one of them can bind to β-sheet binding dye, Congo red and exhibit a minima at 220 nm for CD spectra (17).

One of the denaturing conditions provided to trigger amyloid nucleation is agitating the protein solutions (18, 19). Once the amyloid seeds are formed, the aggregates mature rapidly without any detectable lag phase during the incubation period. Agitation of proteins has been reported to form amyloids of very distinct morphologies (19). In the present study, we agitated ovalbumin to induce formation of amyloid fibrils. However, to our surprise, the aggregate obtained upon shaking for 24 hrs exhibited a nanosheet-like morphology instead of fibrillar form. This is in analogy with a recent report where shaken but not stirred peptoid monolayer was found to form free floating, stable nanosheets (20). Since nanosheets were formed in an amyloid inducing ambience, we investigated their amyloidal character. The various biophysical studies ascertained amyloid character of nanosheets. Further, the nanosheets were tested for release of the precursor proteins and whether the released precursors carry native antigen conformation. The amyloid nanosheets were evaluated for their potential to evoke immune response. The antibodies evoked upon nanosheet immunization were tested for cross-reactivity with native antigen. We anticipated that the availability of both obverse and reverse surfaces in nanosheets may facilitate high drug loading. Therefore, we examined if the anticancer drug, doxorubicin, can be loaded onto nanosheets and later be released. Next, we tested if the amyloid nanosheet formation is a characteristic of only very amyloidogenic proteins like albumins or amyloid nanosheets can be formed by other proteins/peptides especially cell surface antigens as they are the potential targets of immunotherapy. The nanosheets being whole protein bodies can be exploited as antigen delivery system themselves and their free obverse and reverse surfaces can provide ample area for additional drug loading, hence, these nanosheets can emerge as a novel class of biomaterials with applications in simultaneous immunotherapy and chemotherapy. To the best of our knowledge, this report elucidates discovery of amyloid-like nanosheets for ovalbumin and also illuminates the immune responses evoked upon immunizing amyloid nanosheets, firstly.

Results

Continuous shaking of ovalbumin at neutral pH forms amyloid nanosheets

Fluorescence intensity was monitored to detect aggregation in agitated ovalbumin. A marked
increase in fluorescence intensity was observed in the ovalbumin samples kept on agitation (Fig. 1A) as compared to native ovalbumin. The 12 hr and 24 hr aggregates exhibited approx. five-fold and ten-fold increase in fluorescence intensity, respectively, as compared to native form. The 3 hr ovalbumin exhibited only a slight increase in fluorescence intensity than the native form, so chances of aggregation at this state can be ruled out. The turbidity measurements for protein samples agitated for more than 10 hrs also revealed markedly enhanced turbidity in comparison to native ovalbumin, thus giving further evidence in support of aggregate formation (Fig. 1B).

Thioflavin T is a benzothiazole dye that exhibits enhanced fluorescence emissions upon binding to amyloid fibrils and does not bind to amorphous aggregates as well as non-amyloid structures (21, 22). Thus, thioflavin T binding assay was performed to examine amyloid character of aggregates formed by shaking ovalbumin. The agitated protein samples exhibited strong thioflavin T binding as revealed by markedly enhanced thioflavin T fluorescence in comparison to unshaken native ovalbumin (Fig. 1C). The results indicate the formation of β-sheet rich ordered aggregates. Fluorescence microscopy was also exploited to observe thioflavin T binding. The ovalbumin aggregate obtained at 24 hrs of shaking was incubated with thioflavin T and monitored for thioflavin T fluorescence by fluorescence microscopy. The aggregate exhibits a nanosheet like structure with seemingly numerous amyloid seeds in the vicinity (Fig. 1Da). Fig. 1Db shows a mature nanosheet with numerous folds and crumples.

To monitor the formation of amyloid aggregates, Congo red dye is diagnostically used. Amyloid binding to Congo red causes a red shift in the absorbance spectrum of the dye (23). Aliquots of agitated ovalbumin collected at various time points were mixed with Congo red and spectra were obtained. Absorbance intensity was found to increase with increase in agitation time, however, a red shift starts to appear only from the aggregate formed at 12 hrs of agitation. Interestingly, a marked increase in absorption and significant red shift was observed for aggregate formed at 24 hrs indicating β-sheet rich amyloid formation (Fig. 2A). Aggregates obtained upon agitation for more than 24 hrs exhibited negligible change in thioflavin T binding as compared to that observed for 24 hr aggregates (data not shown). This indicates that 24 hr is the saturation point for nanosheet formation and shaking beyond this time does not produce any more sheets.

Once thioflavin T and Congo red binding to the aggregates was ascertained, we also determined secondary structure content of the aggregates by far UV-CD. Native ovalbumin was observed to show double minima at 208 nm and 222 nm which is a hallmark of α-helical forms. On the contrary, 24 hr aggregate exhibited a single negative peak at around 220 nm suggesting their transition to β-sheet conformation (Fig. 2B). The morphology of aggregates formed was further investigated using transmission electron microscopy. The 6 hr and 12 hr aggregates did not reveal any typical amyloid morphology (Fig. 2Ca, 2Cb), however, the aggregates obtained at 24 hrs exhibited a very unique sheet-like morphology (Fig. 2Cc,d). The sheets are seen to be crumpled and having numerous folds.

We also calculated nanosheet yield in the 24 hr aggregates. Nanosheet yield was found to be 67.6%. This indicates that 67.6% of the monomeric ovalbumin converted to nanosheets and rest made the un-polymerized ovalbumin pool.

Nanosheets release precursor proteins having native antigen conformation

In our previous study (16), we suggested exploitation of amyloids for vaccine development considering their property to act as depots for release of precursor proteins and resistance to enzymatic degradation that extends their time in circulation. We wondered whether the nanosheets also release precursor proteins like those observed for fibrils, therefore, we evaluated release of precursor ovalbumin from amyloid nanosheet over a period of 15 days. Interestingly, the ovalbumin nanosheets were found to release the precursor proteins in a time dependent manner and were found to be superior depots for the protein release as compared to fibrillar form of ovalbumin (Fig. 3A). This might have happened as the sheets may be formed of laterally assembled fibrils and several fibrils may be releasing the precursors from their termini in the nanosheets. The fibrillar
ovalbumin although released the precursor ovalbumin in a sustained manner over a period of 15 days but the amount released was lower than that observed for nanosheet at each time point.

In order to analyse the state of released precursors from the nanosheet depot, we performed size exclusion chromatography of the released proteins. Both fibrillar and nanosheet forms of ovalbumin could be seen to release monomers, dimers and higher order oligomers (Fig. 3B). However, the dominant species that is released is monomeric form. It may be possible that oligomeric forms were first released which subsequently released monomeric forms but we did not get into these details as our concern was whether the precursor proteins are released or not. The released precursor proteins, whether in monomeric/dimeric/multimeric forms, may serve the purpose of boosting immune system time and again.

We believe that similar to existence of fibril-monomer equilibrium in conditions of fibril depolymerization, a dynamic equilibrium between the released monomers and nanosheets is also established. Concomitantly, we characterized the nanosheet dissociation equilibrium by calculating $t_{1/2}$, $K_d$, and $K_{off}$ of the nanosheets. The $t_{1/2}$, $K_d$ and $K_{off}$ of nanosheets are found to be 50 days, 3.3 µM and 1.64 x $10^{-7}$ s$^{-1}$ respectively (Table 1). The details of these are provided in the Materials and Methods section.

Next, we studied the nature of the precursor proteins released from nanosheets. Intriguingly, as is observed for fibrillar amyloids, the released precursors from amyloid nanosheets mimicked native ovalbumin for light scattering and thioflavin T binding (Fig. 3C and D). Moreover, the CD spectra of released precursors was found to be similar to those obtained for native ovalbumin (Fig. 3E and F). These results indicate that the released ovalbumin from the nanosheet depot may be refolding to native conformation. It is interesting to observe that the near UV-CD spectrum of released monomers, which indicates tertiary structure of proteins (24), is quite similar to that obtained for native ovalbumin. This demonstrates that not only the secondary structures (as observed from far UV-CD spectrum) but the tertiary conformation of the released monomers is also conserved. It has been previously observed that there is a marked difference in the near UV-CD spectrum of disulphide reduced and disulphide intact (native) ovalbumin (25). Reckoning with this, native-like near UV-CD spectrum of released ovalbumin monomers, in the present study, can be said to suggest conservation of native conformation along with native disulphide bond.

**Immunization with nanosheets induces high antibody titres against native antigen**

Balb/c mice were immunized with ovalbumin nanosheets and the immune response evoked was evaluated. Intriguingly, nanosheets were found to induce significantly higher antibody titres compared to that induced by native ovalbumin (Fig. 4A). The antibody titres induced by nanosheets were even higher than those induced by native ovalbumin given in CFA. Of note, the strong antibody responses evoked against nanosheets are without co-administration of any additional adjuvant. As the native form of ovalbumin did not produce a strong antibody response, hence, the self-assembly of ovalbumin to amyloid nanosheet structure can be said to be responsible for the robust antibody responses. This indicates that amyloid nanosheets have self-adjuvanting property as well.

Nanosheet antisera was also found to react with ovalbumin monomers released from nanosheets. There was practically insignificant difference between reactivity of anti-nanosheet antibodies with intact nanosheets and released monomers. This indicates that both the multivalency of the sheets and released precursors might be contributing to the overall immunogenicity of the nanosheets. Our purpose of exploiting nanosheet as vaccine candidate is fulfilled whether the host system favors either multivalency of the sheets or the released precursors for generating immune response. Whatever be the source of immunogenicity, as long as nanosheets are able to evoke immune response against native antigen, the purpose of exploiting them as vaccine candidates is accomplished.

The cross-reactivity of the nanosheet antisera with native antigen was also tested. The nanosheet antisera were found to be strongly
cross reactive with native ovalbumin (Fig. 4B), indicating that nanosheet immunization is capable of eliciting high antibody titres against native ovalbumin, by virtue of retaining native antigen epitopes and/or releasing precursors carrying native epitopes.

**Precursors released from nanosheets are reactive to both polyclonal and monoclonal anti-native ovalbumin antibodies**

Precursors released from nanosheets were analysed for reactivity with anti-native ovalbumin antibodies. Interestingly, the released precursors from nanosheets were found to react with anti-native ovalbumin polyclonal antibodies (Fig. 4C). This indicates that the released precursors might be harboring native antigen epitopes. To test the conformational integrity of released precursors, we tested reactivity of released ovalbumin with 2D11 monoclonal antibody. This antibody reacts with native ovalbumin but not aggregated forms (Fig. 4D) which indicates that it recognises a conformational epitope. The precursor ovalbumin released from nanosheets was found to be reactive to 2D11 antibody (Fig. 4E) indicating retention of native ovalbumin conformation owing to the possible presence of conformational epitope(s).

The biophysical and immunological results described above indicate that released precursors from nanosheet depot may harbour native protein conformation.

**Immunization of Balb/c mice with nanosheets induces a Th2 biased immune response**

The nature of the immune response was determined by evaluating the isotypes of the responding antibodies in nanosheet immunized group. IgG1 was found to be the dominant isotype with lower levels of IgG2a which supports elicitation of a predominantly Th2 biased immune response (Fig. 5A and B). We also evaluated levels of pro-inflammatory cytokines IFN-γ and IL-12 and anti-inflammatory cytokine IL-4 in order to examine whether any inflammatory response is also evoked upon nanosheet immunization. Nanosheets were found to produce negligible levels of IFN-γ and IL-12 in nanosheet immunized mice in comparison to animals immunized with native ovalbumin (Fig. 5C and D). On the contrary, IL-4 level was found to be significantly higher in nanosheet immunized animals than mice immunized with native ovalbumin (Fig. 5E). This establishes a Th2 biased response and indicates that amyloid nanosheets are non-inflammatory despite being immunogenic.

**Immune response to ovalbumin amyloid nanosheets in C57BL/6 mice**

In order to ascertain the results of the immune response to ovalbumin amyloid nanosheets in Balb/c mice, which is prototypical Th2 mouse strain, we evaluated the immune response to nanosheets in C57BL/6 mice (a prototypical Th1 mouse strain) as well. Similar to that observed in Balb/c mice, a robust antibody response is evoked upon immunizing C57BL/6 mice with amyloid nanosheets (Fig. S1A). In this case as well, nanosheets were found to evoke higher antibody titres than native ovalbumin without co-administration of any adjuvant, confirming the self-adjuvanting property of amyloid nanosheets. Practically similar levels of IgG2a to IgG1 ratio and pro-inflammatory cytokines (IFN-γ and IL-12) are observed in groups immunized with native OVA and amyloid nanosheet (Fig. S1B-D). However, level of anti-inflammatory cytokine, IL-4, is found to be higher in nanosheet immunized group (Fig. S1E). These results indicate that a negligibly inflammatory and Th2 biased immune response is evoked in C57BL/6 mice as well, validating the results obtained in Balb/c mice. Although the pattern for antibody isotypes and cytokines appears to be similar to that observed in Balb/c mice but when we look at the cytokine levels separately in Balb/c and C57BL/6 mice, we observe that levels of pro-inflammatory cytokines are slightly higher and anti-inflammatory cytokines are lower for each group in C57BL/6 mice than that observed in Balb/c mice. This is in agreement with the fact C57BL/6 mouse being a prototypical Th1 mouse strain may favour generation of higher pro-inflammatory response than Th2 prototypical strain of Balb/c mice.

**Nanosheets interact with anticancer drug doxorubicin and also act as depots for its slow and sustained release over time**

Nanosheets were incubated with doxorubicin and were monitored for possible interaction. An increase in absorption intensity of doxorubicin and a red shift indicates interaction of nanosheets with doxorubicin. Moreover, quenching of fluorescence intensity of
doxorubicin upon incubation with nanosheets also indicates possible binding of doxorubicin to nanosheets (Fig. 6A, B).

The release of doxorubicin loaded onto nanosheets was also monitored. Doxorubicin was found to be released from the sheets in a slow and sustained manner over time at pH 7.4 as well as pH 5.0 (Fig. 6C). However, the release was found to be significantly higher at pH 5.0.

Overexpressed tumor antigens form amyloid nanosheets

Overexpressed tumor antigens represent attractive targets for immunotherapy. We selected antigens overexpressed in several cancers and examined their tendency to form amyloid nanosheets. We chose tandem repeat fragment of mucin 1 (MUC1: VTSAPDTRPAAGSTAPPAHG) (26), glypican-3: FVGEFFTDV (27) and CD45: KFLDALISL (27) and analysed amyloid nanosheet formation by these. MUC1 failed to form nanosheets even after 15 days of agitation in all the three pH conditions (pH 2, pH 7 and pH 10). Also, it did not show any sign of aggregation despite addition of 1 M NaCl (Fig. S2A). Interestingly, glypican-3 formed nanosheets after 3 days of agitation at neutral pH in high salt concentration (1 M NaCl) (Fig. S2B). Glypican-3 samples which were not agitated could not form amyloid nanosheets (data not shown). CD45 also could not form nanosheets despite vigorous shaking and addition of concentrated salts but it did form oligomeric aggregates (Fig. S2C).

MUC-1 peptide might have failed to form amyloids because of the presence of numerous proline residues in its sequence which have been reported to inhibit β-sheet formation (28, 29). If we look at the MUC-1 sequence, we find that 25% of the residues are proline which may have developed kinks in the peptide, therefore, instead of forming β-sheets they would have had formed β-turns. Of note, MUC-1 sequence lacks aromatic amino acids as well, that might have further contributed to their inability to aggregate. It is suggested that presence of aromatic residues promotes aggregation as a result of π-π stacking interactions between benzene rings (30).

The rich presence of amino acids such as valine, threonine, phenylalanine and tyrosine which have been reported to favor aggregation (30, 31) might be the reason for easy aggregation of glypican-3 into nanosheets upon shaking. On the other hand, seemingly lesser number of such amino acids in the CD45 peptide may have prevented formation of nanosheets. Intriguingly, coupling of a tripeptide KFF to the CD45 peptide sequence induced formation of nanosheets under continuous agitation for 2 days at neutral pH (without addition of salts) (Fig. S2D). KFF is composed of amino acids having a predilection for aggregation. The peptide or protein sequences reported to form extended 2D nanostructures are phenylalanine and tyrosine rich (32-34). Although, it would be highly premature to say that these amino acids have propensity to form nanosheets, but such reports and results of the present study certainly indicate that these amino acids may be of importance in inducing nanosheet formation. None of the peptides could form nanosheets when agitated at pH 2 and pH 10 (data not shown).

Like glypican-3, KFF containing CD45 peptide (KFF-CD45) could not form nanosheets in the absence of shaking (data not shown). Moreover, nanosheet formation could be observed only at neutral pH irrespective of the protein/peptide taken. In light of these results, it appears that shaking at neutral pH plays a critical role in inducing amyloid nanosheet formation. Despite being composed of amino acids having propensity to induce aggregation, the inability of these peptides to form amyloid nanosheets in the absence of agitation at neutral pH indicates the importance of shaking and neutral pH conditions in inducing amyloid nanosheet formation.

Moreover, nanosheets formed by both glypican-3 and KFF-CD45 showed a marked increase in fluorescence upon thioflavin T binding indicating abundance of β-sheet rich structures (Fig. S2E).

Discussion

Amyloidal structures are conventionally fibrillar in appearance but for several proteins/peptides, whether naturally amyloidogenic or designed amphiphilic, many non-fibrillar amyloids have also been reported
These non-fibrillar amyloids have been exploited for catalysis, biosensing, retroviral gene transfer and bio-based electronics (15, 44-47). In the present study, ovalbumin dissolved in PBS (pH 7.0) was kept on continuous agitation so as to induce formation of amyloid fibrils. However, after 24 hrs of incubation, the aggregates obtained exhibited a unique nanosheet-like morphology instead of fibrils (Fig. 2C). Fine nanosheets with folds and crumples could be observed but their maturation was preceded by unordered oligomeric aggregates as observed in Fig. 2C. The thickness of nanosheet was found to be 5.2 nm by atomic force microscopy (data not shown). Sanii et al. have reported that shaking plays an important role in the generation of protein nanosheets (20). It is believed that the careful motion of shaking allows compression from the air-water interface to precisely fold the protein into desired shape. During the shaking process; the proteins compress, pushing chains of the proteins together and squeezing them into a nanosheet. This process is not possible without the air-water interface, which is why the same effect is not observed when protein solution is stirred (20).

As the nanosheets were observed in conditions usually exploited for the generation of protein fibrils and during the nanosheet maturation, some of the oligomers seen were similar to those conventionally observed in the fibril maturation pathway; we wondered if the nanosheets too bear amyloidial features. Various dye binding assays (thioflavin T and Congo Red), spectroscopic and CD studies (Figs. 1 and 2A,B) indicate the presence of amyloid character in the in-house synthesized nanosheets. The tendency of the nanosheets to carry high β-sheet content similar to amyloid-like aggregates was interesting but not surprising since it has been lately reported that an amyloid (1-40) solution rich in coil, turn, and α-helix but poor in β-sheet developed monolayers with a high β-sheet content when spread at the air-water interface (48). Initially shaking can cause structural instability in the protein inducing it to aggregate with structural transition from α-helix to β-sheet as is conventionally seen with most of the protein aggregation reactions. Then, the aggregating proteins may coalesce and due to continuous agitation the side chains of coalescing aggregates can compress at the air-water interface pushing them into β-sheet rich nanosheets.

Interestingly, nanosheets provided depot effect and released native-like precursors in a manner similar to that observed for fibrils. The precursors released from nanosheets mimicked native protein conformation and appear to be predominantly monomeric in nature (Fig. 3). Therefore, like amyloid fibrils, amyloid nanosheets may also follow thermodynamic equilibrium conditions between nanosheets and released monomers. Considering that the monomer release from nanosheets follows a first order reaction (as detailed in Materials and Methods section), we calculated $K_{off}$ and $t_{1/2}$ of the reaction. Lower $t_{1/2}$ and higher $K_{off}$ of nanosheets as compared to fibrils corroborated the markedly higher release of precursors from nanosheets in comparison to fibrils (Table 1). The $K_{off}$ of nanosheets $(1.6 \times 10^7 \text{ s}^{-1})$ suggests that nanosheets release monomers approximately ten times faster than fibril $(7.5 \times 10^8 \text{ s}^{-1})$. The nanosheet-monomer equilibrium is of particular importance for the proposed immunotherapeutic application, because nanosheets must be both stable enough to guarantee a long duration of precursor release to boost immune system and the release of precursors must be sufficient to evoke desirable immune response. In human subjects, this equilibrium may shift in forward direction as the monomers released mimic native proteins and therefore may get degraded by the proteolytic machinery of the body. Hence, the half-life of nanosheets in vivo may be lower than in vitro half-life. So, it can be concluded that like any other amyloidal aggregate, nanosheets also have a particular half-life and after a certain period of time the whole nanosheet may fully disassemble to monomers. Considering this fate, fibrillar and oligomeric amyloids of gonadotrophin releasing hormone and insulin respectively have been proposed as drug-depots (49, 50). The storage of pituitary hormones as amyloids (4) is the best example of nature’s choice to use amyloids as natural reservoirs for peptide release. It also elucidates physiological tolerance to amyloids. Moreover, there are numerous other evidences that suggest that amyloid based biomaterials are well tolerated in vivo (51-56).

Finding ovalbumin nanosheets to bear amyloid character and capable of releasing precursor
proteins, we evaluated immune response evoked upon immunizing the same. In a manner similar to conventional amyloids, they may also withstand proteolytic degradation which may increase their residence time in circulation. We evaluated immune response to nanosheets with a quest that if they are found to evoke robust immune response, they can be exploited as antigen delivery system for vaccine development. Moreover, keeping in mind that the extended 2D structure of nanosheets may facilitate loading of small-molecule drugs, we analysed loading of anticancer drug, doxorubicin, onto nanosheets. We set forth with the idea that if nanosheets are immunogenic and can also load small-molecule drugs, they can present a modular platform for the development of a novel simultaneous immunotherapy and chemotherapy system with potential clinical applications.

Nanosheets were found to evoke robust immune response and interacted with doxorubicin efficiently. Interestingly, robust antibody responses against self-assembled ovalbumin nanosheets are evoked without co-administration of any additional adjuvant (Fig. 4A), suggesting that nanosheet amyloids have self-adjuvanting property. The strong cross-reactivity of nanosheet antisera with native ovalbumin (Fig. 4B) demonstrates that nanosheet immunization is capable of eliciting high antibody titers against native ovalbumin, by virtue of retaining native antigen epitopes and/or releasing precursors carrying native epitopes. To obtain a clearer picture, ELISA plates coated with precursors released from nanosheets were probed with native ovalbumin antisera (polyclonal antibodies). Intriguingly, released precursors recognized anti-native ovalbumin polyclonal antibodies (Fig. 4C) indicating possible conservation of native ovalbumin epitopes in released precursors. This was further confirmed by probing plates coated with released ovalbumin with 2D11 monoclonal antibody that recognizes native ovalbumin but not denatured ovalbumin. The ovalbumin precursors released from nanosheets reacted with 2D11 monoclonal antibody but the nanosheet itself did not recognize 2D11 antibody (Fig. 4D and E). This indicates a possible conformational change in released precursors, from amyloid state to native form. This phenomenon reiterates our previous finding that native form of proteins may be released from amyloid depots (16). It further elucidates that this phenomenon is independent of the morphological structure of amyloids; whether they are in oligomeric form, fibril or nanosheet form, they may release precursors harboring native antigen epitopes. In other words, release of native protein may be a generic feature of amyloids.

Considering the levels of IgG1 and IgG2a isotypes in responding antibodies and pro- and anti-inflammatory cytokines evoked (Figs. 5 and S1), it appears that nanosheets elicit predominantly Th2 biased immune response with robust humoral and insignificant inflammatory responses. This is in contrast to our previous finding where we found that the oligomeric intermediates formed in the process of fibril maturation evoked Th1 biased inflammatory immune responses (16). It indicates that the mature amyloids like fibrils and nanosheets may evoke Th2 biased responses while the intermediates formed in the process of aggregate maturation may evoke Th1 biased inflammatory responses. Interestingly, Aβ oligomers but not fibrils also have been reported to initiate inflammatory responses (57, 58). This gives a platform to tune the immune response, as per requirement. If Th2 biased immune response is desired, mature amyloids (fibrils and nanosheet) can be exploited on the other hand for evoking Th1 biased immune response, oligomeric intermediates are seemingly better suited. Nevertheless, further immunological studies with amyloid nanosheets formed by other peptides/proteins are needed to come to any generalized statement.

Novel medical applications of 2D nanomaterials in drug delivery, vaccine protein delivery and wound healing, etc. has attracted scientific community across the globe (59-62). 2D nanomaterials are thought to be superior drug delivery agents because of the availability of both reverse and obverse surfaces. Synergistic combination therapy using 2D nanomaterials is also one of the hot areas of research these days (63, 64). However, developing multifunctional 2D nanomaterials with high loading capacity remains a challenge. We propose exploiting the in-house produced amyloid nanosheets as a multifunctional antigen and drug delivery vehicle. Amyloid nanosheets being whole protein bodies capable
of releasing precursor proteins and evoking strong antibody response may themselves act as antigen and delivery vehicle simultaneously. The antigen does not need to be loaded onto the sheets, therefore, the in-house synthesized nanosheets may load small-molecule drugs with high loading capacity. In the present study, we evaluated loading of anticancer drug, doxorubicin, onto ovalbumin amyloid nanosheets. Doxorubicin was found to be successfully loaded onto the sheets and subsequently be released (Fig. 6). This suggests that nanosheets can be used for combined immunotherapy and chemotherapy approaches where the vehicle itself (nanosheets) may serve as immunotherapeutic agent and loaded drug may act as chemotherapeutic agent. Moreover, as the antigen itself makes the vehicle, the other drug(s) can have high loading capacities which is one of the biggest challenges with combined synergistic drug delivery platforms currently in vogue. Fig. 7 shows a schematic illustration of the proposed amyloid nanosheet based dual-delivery vehicle with applications in simultaneous immunotherapy and chemotherapy.

As we propose exploitation of amyloid nanosheets for vaccine development, it becomes indispensable to examine whether amyloid nanosheet formation is limited to amyloidogenic proteins like albumins or it can be extended to real world antigens especially cell surface antigens which are the preferred targets for immunotherapy. Considering these facts, we evaluated formation of amyloid nanosheets by MUC1; a glycosylated transmembrane protein overexpressed in numerous cancers (26), glypican-3; a cell-surface protein overexpressed in hepatocellular carcinoma (27) and CD45; a cell surface leukocyte common antigen overexpressed in leukemia and lymphoma (27). The reason behind studying these antigens for nanosheet formation is their overexpression in various cancers that makes them potential targets for immunotherapy. Interestingly, we could successfully make nanosheets for glypican-3 under continuous agitation at neutral pH in the presence of concentrated salt. MUC1 failed to aggregate despite vigorous shaking and addition of concentrated salts at all the three pH conditions. CD45 also could not form nanosheets despite vigorous shaking and addition of concentrated salts but it did form oligomeric aggregates (Fig. S2). However, when KFF, a tripeptide containing amino acids that favour aggregation, was coupled to CD45 peptide, it successfully formed nanosheets when agitated at neutral pH. Interestingly, unshaken glypican-3 and KFF-CD45 could not form amyloid nanosheets (data not shown). Moreover, in each case, nanosheet formation could be induced only at neutral pH. These results indicate that a confluence of factors such as aggregation inducing amino acids, continuous agitation, neutral pH and ionic strength may be required to induce the formation of nanosheets, although, the conditions may vary for different peptides/proteins. The results also show that nanosheet formation is not limited to amyloidogenic proteins like albumins but it can also be extended to other proteins including cell-surface proteins which is important for developing real-world immunoprophylactic/immunotherapeutic platforms.

Short fibrillizing peptides capable of self-assembling to β-sheet-rich nanofibers have been reported (51). It is feasible to add desired amino acid sequences to the N-terminus of these fibrillizing peptides which self-assemble to form fibrils of the desired peptides. In a manner similar to short fibrillizing peptides, we believe that short nanosheet forming peptides can be designed which could self-assemble to form amyloid nanosheets at specific pH and salt concentrations. Desired peptides can be added to the nanosheet forming peptides which will induce formation of nanosheet of the whole amino acid stretch even if the desired peptide is non-amyloidogenic. Although we believe that amyloid nanosheets can be formed for any peptide/protein under specific agitation and pH conditions irrespective of their amyloidogenic nature, designing nanosheet forming peptides will save time, energy and resources. An epitope or even a precise combination of epitopes can be added to the N-terminus of nanosheet forming peptides leading to development of a novel class of immunotherapeutic platform.

Conclusion
A nanosheet-like structure formed by continuous shaking of ovalbumin was observed in conditions usually exploited for the synthesis of fibrillar amyloids. The nanosheets were
Amyloid nanosheet as antigen and drug delivery system

found to have amyloidal features. Nanosheets released precursor ovalbumin harbouring native antigen epitopes and preserving native antigen conformation. Immunization with nanosheets evokes strong antibody titres cross reactive with native ovalbumin and the immune response evoked is Th2 biased and negligibly inflammatory. As the obverse and reverse surfaces of nanosheets were free, we evaluated loading of anticancer drug doxorubicin onto them. The amyloid nanosheets were found to interact with doxorubicin and also acted as a depot for its release over time. Reckoning with these findings, amyloid nanosheets can be exploited as novel class of antigen delivery system owing to their potential to withstand proteolytic degradation and remain in circulation for longer periods. As they can be successfully loaded with small molecule drugs, they can also be useful for combined immunotherapy and chemotherapy. The results of the present study also demonstrate that formation of amyloid nanosheets is not limited to amyloidogenic proteins like albumins but cell surface antigens (such as tumor antigens) can also form amyloid nanosheets under specific conditions.

Materials and Methods

Chemicals and Reagents—All the reagents used were of the highest purity available. Ovalbumin (A2512), thioflavin T, Congo red and doxorubicin were purchased from Sigma Aldrich (St. Louis, MO, USA). IgG2a (R35–95) isotype control was procured from eBiosciences (San Diego, CA). IgG1 and IgG2a isotypes (550487), and cytokines viz. IFN-γ, IL-12 and IL-4 cytokine estimation kits were procured from BD Biosciences, OptEIA (Franklin Lakes, NJ). Anti-ovalbumin monoclonal antibody 2D11 was purchased from Santa Cruz Biotechnology, Inc. All other reagents were of analytical grade and procured from local suppliers.

Nanosheet formation. Ovalbumin was dissolved in 1 ml of PBS (pH 7.0) carrying 0.01% azide at a concentration of 1 mg/ml and incubated at room temperature under continuous agitation at 180 rpm. Aliquots were collected at various time points and pelleted down at 15,000 g for 15 min using Sigma 3K30 (Germany) microcentrifuge. Monomeric ovalbumin did not sediment at 15,000 g and remained in supernatant. The pellet obtained was characterized by light scattering, thioflavin T fluorescence, Congo red binding, CD studies, atomic force microscopy and transmission electron microscopy.

Fibril formation. Ovalbumin was dissolved in PBS (pH 7.0) carrying 0.01% azide at a concentration of 1 mg/ml and incubated at room temperature under continuous agitation at 90 rpm for 15 days (16). The sample was centrifuged at 15,000 g for 15 min and the pellet was collected and analysed for fibril formation by thioflavin T fluorescence, Congo red binding and transmission electron microscopy (data not shown).

Rayleigh Scattering measurements. Rayleigh scattering measurements were performed on a Hitachi F-4500 fluorescence spectrophotometer at room temperature in a 1 cm path length cell. Samples were excited at 350 nm and spectra were recorded in the range of 300–400 nm. Both excitation and emission slits were fixed at 5 nm.

Turbidity measurements. The turbidity of the incubated samples collected at various time points was monitored by UV absorbance at 350 nm using a Perkin Elmer UV-visible spectrometer model λ25 in a cuvette of 1 cm path length at room temperature.

Thioflavin T binding. Various ovalbumin aggregates (100 µg) were incubated with 30 µM thioflavin T solution (30 µl of 1 mM thioflavin T stock solution) and fluorescence was measured after 30 min on Hitachi F-4500 fluorescence spectrophotometer. Thioflavin T fluorescence was excited at 450 nm and spectra were recorded from 460 nm to 560 nm. The excitation and emission slit widths were fixed at 5 nm and 10 nm respectively. Thioflavin T solution (30 µM) in PB (5mM, pH 7.4) was scanned to obtain the thioflavin T only spectrum. Native ovalbumin (100 µg) incubated with 30 µM of thioflavin T was taken as control.

Fluorescence microscopy was also used to establish the amyloid formation by ovalbumin aggregates. Aggregates formed at 24 hrs were incubated with 20 µM thioflavin T for 30 min at room temperature and further transferred onto glass slide to be analysed under...
fluorescence microscope (Axio, HBU 50/AC; Zeiss, Gottingen, Germany).

**Congo red binding.** Congo red solution (20 \( \mu \)M) prepared in PB (5mM, pH 7.4) from 1 mM stock solution of Congo red (prepared in ethanol) was incubated with 100 \( \mu \)g of various aggregates for 30 min at room temperature. UV absorbance was measured from 300-700 nm on a Perkin-Elmer UV-visible spectrometer model \( \lambda 25 \). Congo red solution (20 \( \mu \)M) in PB (5mM, pH 7.4) served as control (for the measurement of the Congo red-only spectrum). Absorbance of 100 \( \mu \)g of native ovalbumin mixed with 20 \( \mu \)M of Congo red solution was also measured.

**CD measurements.** CD measurements were performed on a JASCO spectropolarimeter (J-815). The instrument was calibrated with D-10-camphorsulfonic acid. All measurements were made at room temperature with a thermostatically controlled cell holder attached to a peltier with Multitech water circulator. Spectra were collected with a scan speed of 100 nm/min and a response time of 2 s. Far-UV CD spectra were taken in the range of 200–250 nm range in a cell of 0.1 cm path length with protein samples having final concentration of 200 \( \mu \)g/ml. Near UV-CD spectra were recorded in the range of 250-320 nm.

**Transmission electron microscopy.** Transmission electron micrographs were collected on JEOL transmission electron microscope operating at an accelerating voltage of 200 kV. Nanosheet formation was assessed by applying 6 \( \mu \)l of sample (100 \( \mu \)g) on a carbon-coated copper grid. Excess of fluid was removed after 2 min and the grids were then negatively stained with 2% (w/v) uranyl acetate.

**Atomic Force Microscopy.** An aliquot of aggregates was placed on a Si disc, dried in a nitrogen atmosphere and the samples were analyzed using a contact mode atomic force microscope (Perkin Elmer).

**Nanosheet yield.** The nanosheet yield was quantified by measuring the soluble protein concentration in the supernatant (at 280 nm) of ovalbumin solution agitated for 24 hrs. Ovalbumin solution constantly agitated for 24 hrs was centrifuged at 15,000 x g for 15 mins. The concentration of proteins in supernatant was determined by UV absorption, and the nanosheet yield was calculated by subtracting this value from the initial concentration of monomeric proteins incubated for nanosheet development. We believe the pellet contained only nanosheets as the transmission electron microscopic images did not show presence of any oligomeric or fibrillar aggregates in samples agitated for 24 hrs.

**In Vitro Release Kinetics.** The ovalbumin samples kept for agitation were pelleted at 15,000 g for 15 min and the pellets obtained were washed three times with PB (pH 7.4) and re-suspended in PB (pH 7.4). The kinetics of ovalbumin release in PB (pH 7.4) was monitored spectrophotometrically at 280 nm (16, 49). The supernatant obtained after centrifugation was analysed at various time points for 15 days on PerkinElmer UV-visible spectrometer model \( \lambda 25 \).

**Size Exclusion Chromatography.** Five milliliters of the sample containing ovalbumin released from nanosheet and fibril after 7 days was applied to a 83-cm long column with an internal diameter of 2.3 cm, filled with about 340 ml of preswollen Bio-Gel P10 (Bio-Rad), and equilibrated with 20 mM phosphate-HCl buffer (pH 3.0) containing 18 mM NaCl. The flow rate was adjusted to 35 ml/h and 1.0-ml fractions were collected. Absorbance of the collected fractions was measured at 280 nm using PerkinElmer UV-visible spectrometer model \( \lambda 25 \) and plotted against the elution volume.

**Theoretical treatment of release kinetics of monomers from nanosheets.** In a manner similar to amyloid fibrils, amyloid nanosheets may follow thermodynamic equilibrium conditions between nanosheet and monomers released. Following equation (eq. 1) may be applied to elaborate the monomer release from nanosheets over time:

\[
\text{Nanosheet}_{\text{sol}} \xrightleftharpoons{K_d} \text{Nanosheet}_{\text{agg}} + \text{Monomer} \quad (1)
\]

\[
K_d = \frac{[\text{Nanosheet}_{\text{sol}}][\text{Monomer}]}{[\text{Nanosheet}_{\text{agg}}]} \quad (2)
\]

\( K_d \) is the equilibrium dissociation constant. In a manner similar to that reported for fibril dissociation (49, 65); at equilibrium, the molar concentration of nanosheets may not change practically as comparatively small amount of monomers may be released, so the expression
for $K_d$ reduces to the concentration of monomers at dissociation equilibrium (eq. 3).

$$K_d = \frac{[\text{Monomer}]}{[\text{Dissociated}]} \quad \text{(3)}$$

Monomer release from nanosheets or nanosheet dissociation is governed by first order rate constant, $K_{off}$, as the dissociation reaction depends exclusively on the nanosheet concentration. In order to calculate $K_{off}$ and $t_{1/2}$ (half-life), we obtained the concentration of undissociated or remaining nanosheets at various time points of release kinetics. The concentration of un-dissociated nanosheets was obtained by subtracting the concentration of monomers released at various time points from the initial concentration of nanosheets taken for release kinetics analysis. Natural log concentration of the un-dissociated nanosheets was plotted as a function of incubation time and linear regression analysis was performed. The slope of the plot gave the value of $K_{off}$ and $t_{1/2}$ was calculated using the equation, $t_{1/2} = \ln 2/K_{off}$.

**Animals.** Inbred female Balb/c mice and C57BL/6 mice (6–8 weeks old, 20±2 g) were obtained from the Animal House Facility of the Interdisciplinary Biotechnology Unit, AMU and Department of Biochemistry, JNMC, AMU, respectively. The mice were housed in commercially available polypropylene cages and maintained under controlled temperature conditions on a 12-h light/dark cycle and had free access to food and water ad libitum. All the animal experiments were performed according to the National Regulatory Guidelines issued by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). The approval IDs for Interdisciplinary Biotechnology Unit, and Department of Biochemistry, JNMC were 332/CPCSEA and 401/CPCSEA, respectively. All the procedures used for the animal experiments were reviewed and approved by the Institutional Animal Ethics Committee of the Interdisciplinary Biotechnology Unit and Department of Biochemistry, JNMC, AMU.

**Mode and Schedule of Immunization.** Animals were immunized subcutaneously in the lower abdominal region aseptically with 50 µg of ovalbumin amyloid nanosheets. A booster was given 3 weeks after the first immunization with 25 µg of the nanosheets.

**Collection of Sera.** The blood was collected from mice after 5 days of last booster. Sera were separated from the clotted blood by centrifugation at 1500 g for 10 min at 4°C. Finally, the supernatant was collected for ELISA analysis and purification of polyclonal antibodies.

**Determination of Antigen-specific Total IgGs by ELISA.** Antigen-specific total IgGs against ovalbumin nanosheet were determined in the sera of mice immunized following the protocol as described elsewhere (16). Briefly, 100 µl (2 µg) of native ovalbumin as well as ovalbumin amyloids (fibril and nanosheet) were dissolved in carbonate/bicarbonate buffer (0.05 M, pH 9.6) and poured into 96-well microtiter plates that were further incubated overnight at 4°C. The plates were then incubated with serially diluted sera at 37°C for 2 h, after the usual washing and blocking steps. Furthermore, the plates were washed again and 100 µl of (1:5000 dilution of stock) horseradish peroxidase-conjugated goat anti-mouse antibodies were added to each well and the plates were incubated at 37°C for 1 h. After the usual plate washing, 100 µl of substrate solution (6 mg o-phenylenediamine) in 12 ml of substrate buffer with 5 µl of 30 % H$_2$O$_2$ was added to the wells and the plates were finally incubated at 37°C for 10 min. The reaction was terminated by the addition of 50 µl of 7% H$_2$SO$_4$. The absorbance was read at 490 nm with a microtiter plate reader (Bio-Rad).

**ELISA of Ovalbumin Released from Nanosheets.** Briefly, 96-well microtiter plates were incubated overnight with 100 µl (2 µg) of ovalbumin released from nanosheets in carbonate/bicarbonate buffer (0.05 M, pH 9.6) at 4 °C. After the usual washing and blocking steps, the plates were finally incubated with serially diluted anti-native ovalbumin-specific polyclonal and 2D11 monoclonal antibodies at 37 °C for 2 h. After washing the plates, 100 µl of (1:5000 dilution of stock) horseradish peroxidase-conjugated goat anti-mouse antibodies were added to each well and the plates were incubated at 37 °C for 1 h. Substrate solution (100 µl) was added to the wells after the usual plate washing, which were finally incubated at 37 °C for 40 min. For terminating...
the reaction, 50 µl of 7% H₂SO₄ was added to the wells. The absorbance was read at 490 nm with a microtiter plate reader (Bio-Rad).

**Determination of Antibody Isotype in Sera of Immunized Mice.** Sera from mice immunized with nanosheets were analyzed for antibody isotypes using the protocol described elsewhere (66). Briefly, 2 µg (100 µl) of antigen in carbonate/bicarbonate buffer (pH 9.6) was added to 96-well microtiter plates that were incubated overnight at 4°C. After washing and blocking steps, the plates were incubated with normally diluted sera at 37°C for 2 h. After excessive washing of the plates, 100 µl of (1:5000 dilution of stock) goat anti-mouse anti-IgG1, and IgG2a antibodies were added in each well and incubated for 1 h at 37°C. Plates were washed again and 100 µl of (1:5000 dilution of stock) horseradish peroxidase-conjugated rabbit anti-goat antibodies were added to each well and each plate was incubated at 37°C for 1 h. After further washing of the plates, 100 µl of substrate solution was added to the wells and the plates were finally incubated at 37°C for 40 min. The reaction was stopped by the addition of 50 µl of 1 M H₂SO₄. The absorbance was read at 490 nm with a microtiter ELISA plate reader (Bio-Rad).

**T Lymphocyte Isolation from Spleens of Immunized Mice.** Mice immunized with native as well as ovalbumin amyloids (fibril and nanosheet) were sacrificed post day 5 after the last booster. T lymphocytes were isolated from the spleens of sacrificed mice as described elsewhere (66). Briefly, spleens isolated from animals belonging to various immunized groups were macerated and suspension was treated with ACK lysis buffer (0.15 mol/liter of ammonium chloride, 10 mmol/liter of potassium/bicarbonate, and 88 mmol/liter of edetic acid) for lysis of the red blood cells. The cell suspension was centrifuged at 1500 g for 5 min, and the cell pellet was washed with Hanks’ balanced salt solution 3 times and re-suspended in RPMI 1640 medium containing 10% fetal calf serum and 0.1% antimycotic mixture.

**Cytokine Assay: Determination of IFN-γ, IL-4 and IL-12 by Sandwich ELISA.** Levels of IFN-γ, IL-12 and IL-4 cytokines induced by lymphocytes upon their culture in the co-presence of ovalbumin nanosheet were quantitated using appropriate and specific biotinylated antibody pairs according to the manufacturer’s protocols. Briefly, 96-well microtiter plates were coated with 50 µl of the purified capture antibodies in carbonate/bicarbonate buffer (pH 9.5) at 4°C. After the usual washing and blocking steps, 50 µl of the supernatant (isolated from cultured splenocytes after 48 h) was poured in each well for determining the level of cytokine induced. Plates were washed and incubated with biotinylated polyclonal goat anti-mouse cytokine detection antibody. Furthermore, after washing the plates, 100 µl of streptavidin-horseradish peroxidase conjugate was added to each well and plates were incubated for 30 min at room temperature. The plates were then washed and the colored complex was developed with tetramethylbenzidine. The absorbance was read at 450 nm with a microtiter plate reader (Bio-Rad). A known specific recombinant cytokine was used as standard for calculating the level of the given cytokine in the samples tested, and the concentration was expressed as pg/ml.

**Interaction of ovalbumin amyloid nanosheets with anticancer drug doxorubicin.** 2 ml of doxorubicin (2.5 mg/ml) aqueous solution was added to 3 ml of ovalbumin nanosheet (50 µg/ml) solution in water. After stirring vigorously overnight, the obtained doxorubicin loaded nanosheets were gathered by centrifugation and washed with PBS. Afterwards, the doxorubicin loaded nanosheets were re-dispersed in PBS for further use.

Interaction of doxorubicin with nanosheets was ascertained by recording UV-Vis absorbance spectra and fluorescence intensity. To study the fluorescence quenching of doxorubicin by nanosheets, the fluorescence spectra of doxorubicin loaded nanosheets were measured on a fluorescence spectrophotometer (FL-4600, Hitachi, Japan), using free doxorubicin (47.5 µg/ml) as control. To exclude the inner filter effect, nanosheet (50 µg/ml) and doxorubicin (47.5 µg/ml) were mixed for only ten seconds, and then the fluorescence spectrum was measured instantly.

**Release of doxorubicin from nanosheets.** Doxorubicin loaded nanosheets were suspended in 10 ml of PBS (pH 7.4) and were poured in a dialysis bag. Then the dialysis bag was placed in 50 ml of PBS. The whole
assembly was maintained at 37 ± 0.5 °C, covered by parafilm to avoid evaporation and shaken at 90 cycles/min. At fixed time intervals, 2 ml of medium was withdrawn and replaced with the same volume of fresh buffer to maintain the required sink condition. This was taken into account while calculating cumulative drug release. The sample was filtered and drug quantity in filtrate was determined by HPLC analysis.

**Peptide synthesis.** Tumor peptides were synthesized following the published protocol (67) with slight modifications in our laboratory. Briefly, peptides were synthesized manually via the solid phase method on Wang resin utilizing standard Fmoc chemistry. The peptides were purified by reverse phase HPLC on an analytical Waters Symmetry C18 column using a linear gradient of 20–80% acetonitrile for 40 min with a flow rate of 0.6 mL/min. Both acetonitrile and water contained 0.1% trifluoroacetic acid. The purity of the peptides was further determined to be ≥95 by reverse phase analytical chromatography.

**Nanosheet formation by overexpressed tumor antigens.** Overexpressed antigens represent attractive targets for immunotherapy. We exploited tandem repeat fragment of mucin 1 (MUC-1), glypican-3 and CD45 having sequences VTSAPDTRPAGSTAPPAHG (24), FVGEFFTDV (25) and KFLDALISL (25) respectively for examining nanosheet synthesis. The in-house synthesized peptides were dissolved in buffers of pH 2, pH 7 and pH 10 (with or without 1 M NaCl) and kept under continuous agitation for various time periods. Aggregates obtained were characterized by thioflavin T binding and transmission electron microscopy using the protocols described in this paper.

**Statistical Analysis.** Data were analyzed and two groups were compared using Student’s t test and one-way analysis of variance (Holm-Sidak method) was used to compare all groups to each other. p values <0.05 were considered significant. SigmaPlot (versions 10 and 11; SigmaPlot software, San Jose, CA) software was used for data presentation.

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**Conflict of Interest Statement**
Authors report no conflict of interest.

**References**


Amyloid nanosheet as antigen and drug delivery system


**FOOTNOTES**

The abbreviations used are: CD, circular docherism; CFA, complete Freund’s adjuvant; ELISA, enzyme-linked immunosorbent assay; 2D, two dimensional; IgG, immunoglobulin G; IL, interleukin

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<th>Table 1. Properties of ovalbumin aggregates</th>
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18
Figure 1. Continuous shaking induces amyloid nanosheet formation. A. Rayleigh scattering measurements at 350 nm for various aggregates. B. Turbidity measurements of various aggregates at 350 nm. Error bars exhibit standard error of three independent experiments. C. Thioflavin T binding assay of various aggregates as analysed by fluorescence spectroscopy. Thioflavin T emission spectra were obtained by excitation at 450 nm and emission in the range of 460–560 nm. D. Thioflavin T binding to ovalbumin nanosheets i.e. 24 hr aggregate (100 µg) as visualized by fluorescence microscopy. Thioflavin T (30 µM) was incubated with ovalbumin nanosheets for 30 min. Further, 5 µl of the suspension was placed onto the glass slide and observed under fluorescence microscope. OVA denotes ovalbumin and ThT denotes Thioflavin T. Data are representative of at least three independent experiments with similar observations.
Figure 2. Ovalbumin forms giant β-sheet rich amyloid nanosheets. A. Absorption spectra of Congo red with various aggregates. The aggregates were scanned in the 400–700 nm range. B. Secondary structure determination of nanosheets by UV-CD. Native ovalbumin and nanosheet (200 µg/ml) were scanned in the range of 200–250 nm. Data are representative of at least three independent experiments with similar observations. C. Nanosheet formation as seen by transmission electron microscopy. (a) 6 hr ovalbumin (b) 12 hr ovalbumin (c) and (d) 24 hr ovalbumin (nanosheet). Various aggregates (100 µg) were coated onto the grid. OVA denotes ovalbumin and CR denotes Congo red. Images shown are those selected after similar observation of at least two independent experiments performed for each sample.
Figure 3. Nanosheets release precursor proteins over an extended time period and the released precursors bear native ovalbumin properties. A. In vitro release kinetics of ovalbumin from amyloid nanosheets over a period of 15 days as determined by taking absorbance at 280 nm. B. Size exclusion chromatography profile of released ovalbumin after 7 days exhibiting release of monomers, dimers and oligomers with monomers being the predominant species. C. Light scattering by ovalbumin precursors released from nanosheets at 350 nm. D. Thioflavin T binding assay of ovalbumin released from nanosheets. E. Secondary structure determination of released precursors by far UV-CD. F. Tertiary structure determination of released precursors by near UV-CD. OVA-R (Fibril) and OVA-R (Nanosheet) denote ovalbumin released from fibril and nanosheet respectively. Error bars represent standard error of three independent experiments. At least three independent experiments were carried out for each sample and data obtained with similar results are presented.
Figure 4. A. Nanosheet immunization induced strong antibody titres against nanosheets and ovalbumin released from nanosheets. ELISA plates were coated with native ovalbumin, fibril and nanosheet and allowed to react with antisera obtained from animals immunized with the same. Wells coated with ovalbumin released from sheets were probed with anti-nanosheet antisera. B. Nanosheet antisera is cross reactive to native ovalbumin. ELISA plates were coated with native ovalbumin and allowed to react with antisera obtained from animals immunized with native ovalbumin (both in PBS and CFA), fibril and nanosheet. C. Ovalbumin released from nanosheets are reactive to native ovalbumin specific polyclonal antibodies. ELISA plate was coated with native ovalbumin, ovalbumin released from fibril and nanosheet and allowed to react with antisera obtained upon native ovalbumin immunization. D. 2D11 anti-ovalbumin monoclonal antibody recognises native ovalbumin but not aggregated ovalbumin. ELISA plate was coated with native ovalbumin, fibril and nanosheet and allowed to react with 2D11 monoclonal antibody. E. Ovalbumin precursors released from nanosheets are reactive to 2D11 antibody. ELISA plate was coated with native ovalbumin and ovalbumin released from fibril and nanosheets and allowed to react with 2D11 antibody. Results are presented as mean of three independent experiments with error bars representing standard error of three independent experiments. n.s. denotes ‘not significant’. OVA-R (Fibril) and OVA-R (Nanosheet) denote ovalbumin released from ovalbumin fibril and nanosheet respectively.
Figure 5. Nanosheet immunization induces Th2 biased immune response. A and B. IgG1, IgG2a levels in sera of mice immunized with nanosheets. C, D and E. IFN-γ, IL-12 and IL-4 production in ovalbumin challenged splenocyte cultures. Results are presented as mean of three independent experiments with error bars representing standard error of three independent experiments. n.s. denotes ‘not significant’.
Figure 6. Nanosheets interact with doxorubicin and release it subsequently. A. UV-Vis absorption spectra of doxorubicin and nanosheets loaded with doxorubicin. B. Fluorescence spectra of doxorubicin and nanosheets loaded with doxorubicin. C. Release (%) of doxorubicin from nanosheets at pH 7.4 and pH 5.0. For C, data are expressed as mean ± standard error of three independent experiments. DOX denotes doxorubicin and Nanosheet-dox denotes doxorubicin loaded nanosheets.
Figure 7. Schematic illustration of amyloid nanosheet based simultaneous immunotherapeutic and chemotherapeutic platform. The model shows that native antigen monomers under continuous agitation at neutral pH (in the presence or absence of salts) form nanosheet bearing amyloid-like character. The sheets are capable of releasing monomers predominantly, harboring native antigen conformation. These sheets can be loaded with small-molecule drugs. If drug-loaded sheets are injected, they may act as reservoirs for simultaneous release of native-like antigens and loaded drugs, hence, may have applications in simultaneous immunotherapy and chemotherapy. Immunization with nanosheets leads to generation of native-antigen specific antibodies.
Ovalbumin self-assembles into amyloid nanosheets that elicit immune responses and facilitate sustained drug release

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