Original Research

Nanoallergens: A multivalent platform for studying and evaluating potency of allergen epitopes in cellular degranulation

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Abstract

Degranulation caused by type I hypersensitivity (allergies) is a complex biophysical process, and available experimental models for studying relevant immunoglobulin E binding epitopes on allergen proteins lack the ability to adequately evaluate, rank, and associate these epitopes individually and with each other. In this study, we propose a new allergy model system for studying potential allergen epitopes using nanoallergens, liposomes modified to effectively display IgE binding epitopes/haptens. By utilizing the covalently conjugated lipid tails on two hapten molecules (dinitrophenol and dansyl), hapten molecules were successfully incorporated into liposomes with high precision to form nanoallergens. Nanoallergens, with precisely controlled high-particle valency, can trigger degranulation with much greater sensitivity than commonly used bovine serum albumin conjugates. In rat basophil leukemia cell experiments, nanoallergens with only 2% hapten loading were able to trigger degranulation *in vitro* at concentrations as low as 10 pM. Additionally, unlike bovine serum albumin-hapten conjugates, nanoallergens allow exact control over particle size and valency. By varying the nanoallergen parameters such as size, valency, monovalent affinity of hapten, and specific IgE ratios, we exposed the importance of these variables on degranulation intensity while demonstrating nanoallergens' potential for evaluating both high- and low-affinity epitopes. The data presented in this article establish nanoallergen platform as a reliable and versatile allergy model to study and evaluate allergen epitopes in mast cell degranulation.

Keywords: Allergy, liposome, antigen, degranulation, epitope, immunogenic

***Experimental Biology and Medicine* 2016; 241: 996–1006. DOI: 10.1177/1535370216644533**

Introduction

Type I hypersensitivity is primarily caused by immune recognition of otherwise innocuous molecules, resulting in degranulation reactions in mast cells, releasing histamine, inflammatory cytokines, and other inflammation causing molecules into circulation.[1] Mast cell degranulation is typically triggered by the crosslinking of the high-affinity immunoglobulin E receptor (FcεRI) through multivalent interactions between the allergen-specific FcεRI bound immunoglobulin E (IgE) antibodies and the allergen protein. Here, we describe a new liposome-based synthetic allergen platform—nanoallergens—for stimulating degranulation responses that offer precise control over allergen characteristics such as antigen valency and epitope heterogeneity. The results of this study establish nanoallergens as a potent and versatile platform delivering reproducible outcomes that can be used to elucidate novel intricacies of allergen–IgE interactions and degranulation responses.

The biochemical interactions between allergen and IgE in degranulation responses are typically complex in nature due to multivalent binding events of allergen proteins and competing intracellular pathways. A single allergen molecule binds to multiple IgE antibodies attached to FcεRI receptors causing them to cluster on cell surface.[2–6] The crosslinking of receptors initiates an intracellular cascade that results in degranulation.[7] Until recently, most *in vitro* work on allergic reactions has sought to characterize the IgE-allergen binding, assuming that IgE binding affinity necessarily equates to immunogenicity.[8–11] However, clinical data does not seem to validate this assumption; multiple studies have demonstrated that there is not a direct correlation between allergen-specific IgE binding affinity and clinical response to allergens.[12–15] Likewise, in our laboratory, we have demonstrated the importance of weaker affinity epitope during the degranulation response.[16,17]

This discrepancy between IgE-allergen binding affinity and clinical response is likely due to the complexities that

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arise both from the biological mechanisms of degranulation response and allergen protein structure. Biological factors such as intracellular inhibitory pathways, IgE clonal variability, differences in immunogenic epitope affinities, and relative IgE concentrations in patients make it very difficult to directly assess allergen immunogenicity with current laboratory techniques such as ImmunoCAP ELISA assays.[13,18–21] Additionally, B-cells may or may not produce specific IgEs to individual epitopes on allergen proteins. The number of epitopes and the positions of those epitopes that have a specific IgE will be unique to each patient and drastically affect the apparent allergen protein-IgE complex affinity and therefore the degranulation response.

In cellular-based allergy research, the most commonly used experimental model is a synthetic allergy system using small molecule 2,4-dinitrophenol (DNP) as the hapten (small molecule that elicits an immune response), and a monoclonal anti-DNP IgE (IgE^{DNP}) with rat basophil leukemia (RBL) cells. In order to appropriately simulate RBL cell degranulation *in vitro*, these DNP groups are covalently bonded to bovine serum albumin (BSA) to create a multivalent DNP-BSA allergen that can crosslink IgE^{DNP} and trigger degranulation. Using similar methodology, other hapten–antibody pairs have also been used in degranulation studies. One of the more common is the small molecule dansyl chloride (dansyl).[17,21,22] The hapten-BSA system, while commonly used to trigger degranulation, does not accurately mimic protein allergens. Although the BSA protein has several reactive amine groups, it is difficult to control the specific number of conjugations and their molecular orientation on each individual BSA protein. Importantly, this system also does not reflect the epitope heterogeneity or the polyclonal nature of clinical IgE's, hence is not an appropriate model to simulate and study a natural response.[21,23] Likewise, hapten-BSA conjugates have a limited valency, (~20, given the number of lysines for binding) which restricts their ability to stimulate degranulation with low-affinity peptide mimetics. Given the limitations of the BSA system, a model system for accurate and reliable allergen epitope presentation is urgently needed for successful adaption of *in vitro* allergy research toward clinically relevant allergen proteins.

Our laboratory has recently developed a tetravalent allergy model that can present multiple different hapten molecules on a single flexible polyethylene glycol scaffold that can stimulate degranulation.[17,21,24–26] This design allowed control over the avidity between the allergen molecule to receptor bound IgEs. This system has been exceptionally valuable in studies of IgE-FcεRI clustering and enabled us to demonstrate the significance of weak affinity epitopes in triggering cellular degranulation.[17,26] However, we identified that this system has limited functionality with clinically relevant allergens, given that protein allergens can possess up to 12 epitopes for a single allergen molecule.[23,27] More importantly, natural allergen epitopes, when replicated as short peptide fragments, have a decreased affinity for their associated IgE and typically require a much higher valency to mimic protein allergens in stimulating degranulation at comparable concentrations.

In our laboratory, we have recently developed methods for effective display of different moieties on liposome surfaces.[28–31] The lipids comprising the liposome can be covalently linked with various bioactive molecules such as peptides or small molecules prior to liposome formation, giving precise control over molecule loading. This technique is well established for cancer targeting both *in vivo* and *in vitro*.[29,31,32] Precise control allows us to incorporate as many epitopes as necessary to form highly multivalent nanoparticles with tunable valency, heterogeneity and particle size, making liposomes ideal candidates to present immunoreactive epitopes, and model allergens proteins. In this paper, we demonstrate the utility of the nanoallergens platform using DNP and dansyl nanoallergens. The nanoallergen platform is designed to provide a means to analyze additional aspects of allergens and determine which IgE/epitope interactions carry higher significance for stimulating the degranulation responses.

Materials and methods

Materials

N-Fmoc-amido-$dPEG_6$-acid (Fmoc is also known as fluoren-9-ylmethoxycarbony) was purchased from Quanta BioDesign. *N*-Fmoc-Glu(OtBu)-OH, Boc-Lys(Fmoc)-OH, Fmoc-lys(ivDde)-OH, NovaPEG Rink Amide resin, HBTU [2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate], Fmoc-Arg(pfb)-OH, and BSA were purchased from EMD Biosciences. IgE^{DNP} (clone SPE-7), dansyl chloride, 1-Fluoro-2,4-dinitrobenzene, *N,N*-diisopropylethylamine, trifluoroacetic acid, Triisopropylsilane, hydrazine, cholesterol, dichloromethane, 2-proponol, ACN (acetonitrile), and piperidine were from Sigma and DMF (dimethylformamide) (>99.8%), chloroform, DiD fluorescent dye (3H-Indolium, 2-(5-(1,3-dihydro-3,3-dimethyl-1-octadecyl-2H-indol-2-ylidene)-1,3-pentadienyl)-3,3-dimethyl-1-octadecyl-, perchlorate), Minimum Essential Media were purchased from Thermo Fisher. IgE^{dansyl} (clone 27–74) were purchased from BD Biosciences. DSPC (1,2-distearoyl-*sn*-glycero-3-phosphocholine), DSPE-mPEG2000 (1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt)), membranes, and all mini extruder components were purchased from Avanti Polar Lipids (Alabaster, AL, USA). DNP-BSA conjugate was purchased from Invitrogen.

Statistical evaluation

Unless otherwise stated all error bars represent the standard deviation of triplicates in a single experiment. For degranulation experiments, the data are a representative experiment of several experiments; all others were a single experiment. EC_{50} values and error were calculated using Origin 7 software. All p values were calculated using an unpaired student's t test.

Synthesis of hapten-conjugated BSA molecules

The BSA-dansyl was prepared as previously described.[21] Briefly, BSA at $10\,mg\,mL^{-1}$ in 1 mL of bicarbonate buffer

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(0.1 M, pH 9.0) and 100 μL of 10 mg/mL of dansyl chloride DMF were combined and incubated at room temperature for 2 h. The conjugated BSA was purified using a 0.5-mL 10 kDa molecular mass cut-off spin concentrator (Millipore). RP-HPLC was used to determine purity on an Agilent 1200 series system using a Zorbax C8 poroshell column with a two phase, 90/10 ACN/water and water mix with a flow rate of 2 mL/min at 60°C. The gradient was 5% water to 100% ACN/water mix in 5 min. The dansyl-BSA (elution time 4.8 min) was estimated to be >97%. There were 18 dansyl molecules per BSA as determined by the absorbance ratio of 335 to 280 nm.

Synthesis and purification of lipid-hapten conjugates

The lipid-dansyl and lipid-DNP conjugates were synthesized using Fmoc chemistry on solid support using NovaPEG Rink Amide resin as previously described.[21] The synthetic scheme is described in Figure S–1. Briefly, protected molecules with terminal acid groups were activated with HBTU and a 4-fold molar excess of *N,N*-diisopropylethylamine for 5 min and then conjugated to the resin over 30 min. Fmoc was deprotected with 20% piperidine in DMF, and IvDdE was deprotected using 2% hydrazine in DMF. Deprotection and coupling steps were monitored with Kaiser tests. Lipid-hapten conjugates were cleaved using a 95/2.5/2.5 trifluoroacetic acid/water/Triisopropylsilane solution for 45 min. Lipid-hapten molecules were purified using 1200 Agilent RP-HPLC using a semi-preparative Zorbax C3 column. A two phase water and 70/20/10 IPA/ACN/water mix were used for purification with a gradient of 60–100% IPA mix over 10 min at a flow rate of 3 mL min^{-1}. Hapten-amino acid conjugates were purified using a Zorbax C18 column, using a two phase water/ACN system with a gradient of 20–50% ACN in 10 min. The product was confirmed using a Bruker microTOF II mass spectrometer (Figure S–2). Absorbance peaks at 220 and 280 nm were collected and verified for purity with analytical injections (>95%) (Figure S–3).

Synthesis of hapten-BSA conjugates

Both DNP-BSA and dansyl-BSA conjugates were synthesized as described previously.[21] Briefly, BSA was dissolved at a 10-mg/mL concentration in 4 mL of 50 mM carbonate–bicarbonate buffer (pH 9.6). Then, 1 mL of 200 mg/mL of hapten molecule (dansyl chloride or 1-Fluoro-2,4-dinitrobenzene) dissolved in DMF was added dropwise over 5 min. The mixture was reacted at room temperature for 1 h then purified using a spin concentrator with a 10-kDa molecular weight cut-off to remove unreacted hapten.

Nanoallergen preparation

Liposomal nanoallergens were prepared using a procedure as previously described.[28,29] Briefly, DSPC, mPEG-2000-DSPC, cholesterol, and lipid-hapten conjugates were dissolved in chloroform, lyophilized, rehydrated in PBS at 60°C and then extruded through a 200, 100, 80, or 50-nm polycarbonate filter (Avanti). For some homogeneous nanoallergens (i.e., only DNP or dansyl-lipid loaded), a lipid with an arginine head-group was added at 0.5% of total lipid to ensure particle homogeneity. This lipid followed a similar synthetic scheme as the hapten-lipid conjugates but with the addition of two arginine resides in place of hapten molecules.

Particle characterization

Liposomes were measured for size using dynamic light scattering (DLS) analysis via the 90Plus nanoparticle size analyzer (Brookhaven Instruments Corp.), using 658 nm light observed at a fixed angle of 90° at 20°C. Liposome samples were diluted with 0.22 μM filter sterilized PBS to a 1.25-nM liposome concentration immediately after extrusion, placed in a 50-μL quartz cuvette and particle sized.

Cell culture

RBL-2H3 cells were cultured in Minimum Essential Media (Gibco) with 10% fetal bovine serum (Gemini BioProducts) as previously described.[24] Briefly, every 48 or 72 h RBL cells were trypsinized with 1 mL of trypsin-EDTA solution (Thermo Scientific) for 5 min at 37°C after achieving confluency ($\approx 0.5 \times 10^6$ cells per mL). Then, cells were removed from the plate, and split 1:3 into fresh media.

Degranulation assays

RBL degranulation assays were performed as previously described following the standard beta hexosaminidase assay, expect using nanoallergens as the allergen.[17] These assays were run in triplicate and performed at least twice as independent experiments. Briefly, cells at 150,000 cells per mL per added to a cell culture 96 well plate (0.1 mL per well) and allowed to attach to the plate for 6 h until plate was confluent. RBL cells were then incubated with 1 μg/mL of total IgE overnight prior to nanoallergen incubation using 75% $IgE^{cyclinA}$ as an orthogonal IgE (i.e., an IgE specific to a molecule, cyclinA, which is not used in this study) to simulate physiological conditions for all degranulation assays.[21] The next morning, the plate was washed with Tyrodes buffer (0.2 mL each well, two times), and 5 μL of nanoallergen solution were added into 0.095 mL of Tyrodes in each well. For positive control well, a 1% solution of Triton X was added to lyse cells. After incubating for 1 h, 50 μL of cell supernatant solution were added to 50 μL of 1 mM p-nitrophenyl N-acetyl-β-D-glucosamide solution in citrate buffer (pH 4.5) and allowed to incubate at 37°C for 1 h. This reaction was stopped by adding a 1-mM solution of glycine (pH 10.7) and the absorbance read at 405 nm. To calculate percent degranulation, the absorbance from the triplicate was averaged and then subtracted from the negative control and divided by positive control.

Fluorescence quenching assay

The binding constants for dansyl conjugates were determined as previously described.[26] Briefly, dansyl conjugates were titrated into wells containing 15 nM IgE^{dansyl} and then the fluorescence read at various concentration points (Ex = 280 nm, Em = 335) in triplicates. Because dansyl

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absorbs at around 335 nm, the fluorescence of tryptophan resides on IgEs are quenched, and this drop in signal is correlated to binding.

Flow cytometry

RBL-2H3 cells were plated in 0.5 mL wells for 6 h then incubated with 1 μg/mL of 50%/50% IgE^{DNP}/IgE^{dansyl} overnight. Cells were then washed with 1 mL of Tyrodes buffer containing 0.05 mg/mL BSA to prevent non-specific interactions. Nanoallergens containing 0.5% DiD were added to cells with Tyrodes/BSA buffer, incubated for 5 min at room temperature, washed again with Tyrodes/BSA buffer, quickly scrapped, and analyzed with a Guava EasyCyte flow cytometer (EMD Millipore) in at least two separate experiments.

Kinetic experiments

RBL-2H3 cells were diluted 1 to 3 from a confluent plate then added into a 24 well dish and allowed to adhere to the plate overnight. The cells were then incubated with 1 μg mL^{-1} of 25% IgE^{DNP} using 75% $IgE^{cyclinA}$ as orthogonal IgE to simulate physiological conditions for 24 h. Cells were then placed on ice for 1 h, washed with ice cold Tyrode's buffer containing 0.05 mg/mL BSA to prevent non-specific interactions. Nanoallergens were formed at 50, 100, and 200 nm containing 2% DNP hapten and 5% mPEG2000, and DiD dye is added to ensure 600 dye molecules per liposome. These nanoallergens were added to the wells and incubated for 2–120 min, quickly washed with ice cold Tyrode's/BSA buffer, scrapped, and analyzed with a Guava EasyCyte flow cytometer in at least two separate experiments.

Western blot

RBL cells were plated at approximately 50,000 cells per mL into six well dishes. Then, the cells were washed twice with Tyrode's buffer, incubated at 37°C for 30 min. RBL cells were incubated with varying concentrations of nanoallergens containing an 85/5/5/5 DSPC/HSPC-mPEG200/DNP-lipid/dansyl lipid with 50% cholesterol of total lipid added for 5 min at room 37°C. Following stimulation, cells were washed, scraped, and placed on ice and lysed with 0.5% NP-40 and 0.5% deoxycholate in 4°C phosphorylation solubilization buffer. Samples were normalized with a Bradford assay for total protein content and immune-precipitated using agarose conjugated monoclonal anti-SHIP antibody (P1C1) from Santa Cruz Biotechnology with three subsequent washing steps with phosphorylation buffer containing 0.5% NP-40. Cell lysates were then analyzed with a western blot using anti-p-Tyr antibody (PY99) at a 1:5000 dilution or free anti-SHIP antibody (P1C1) at a 1:1000 dilution from Santa Cruz Biotechnology as previously described.[33] Briefly, cell lysates were boiled with reducing loading buffer (Thermo) for 5 min then run on a 10% SDS-PAGE gel for 1 h. Gel was then blotted onto a nitrocellulose membrane over 1.5 h.Membrane was then washed with Tris-Buffered Saline with 0.05% Tween 20 (TBST), blocked with 5% BSA in TBST for 1 h, incubated with primary antibodies at overnight, washed again, and incubated with secondary HRP conjugated antibody (Goat Anti-rabbit-HRP, Santa Cruz Biotechnologies) for 1 h, then analyzed with analyzed with Western ECL Substrate (Bio-Rad).

Results

Nanoallergen design

In the design of nanoallergens, we used a liposomal functional group display platform that was developed in our laboratory, where the ligands are covalently attached to lipids using appropriate linkers and then purified and characterized prior to incorporating into the liposome formation.[28,29] This platform relies upon covalently linking groups to be displayed multivalently on a liposomal to fatty acid tails with a flexible ethylene glycol linker. These molecules are made, purified, and incorporated unilamellar liposomes through extrusion methods. The two most commonly used haptens in modeling allergy systems are DNP and dansyl, due to their differing monovalent affinities and commercially available specific IgE clones. Anti-DNP IgE (IgE^{DNP}) has a stronger affinity for DNP than anti-dansyl IgE (IgE^{dansyl}) has for dansyl ($K_d^{DNP} = 15$ nM; $K_d^{dansyl} = 147$ nM), making them an excellent pair to study for the effects of varying epitope affinities, and thus making the system physiologically relevant (Table S–1).[17,21,24,34] In order to facilitate hapten presentation on the liposome surface, hapten-lipid conjugates were synthesized using a similar approach previously developed in our laboratory (Figure 1).[21,28,29] The hapten conjugates varied from 0.01 to 10% of total lipid while the remainder of the liposomes consisted of DSPC (Table S–2). For most studies, the nanoallergens consisted of 2% lipid-hapten conjugate unless otherwise specified. Liposomes of 50, 80, 100, and 200 nm diameters were prepared using extrusion methods, and unless otherwise stated, 100 nm diameter particles were used for most studies. We confirmed the particle sizes using DLS analysis (Table S–3). For example, the 100 nm particle had an observed diameter of 110.1 ± 1.4 and 113.5 ± 1.2 nm, respectively, for 2% hapten-loaded nanoallergens for the DNP and dansyl nanoallergens, respectively. The increase in size is likely due to the PEG cloud and haptens presented on the surface of the liposome.

Nanoallergens trigger degranulation using single haptens

We first evaluated the ability of a single hapten system to trigger degranulation using RBL-2H3 cells primed with either IgE^{DNP} or IgE^{dansyl} by using either DNP-lipid or dansyl-lipid loaded liposomes. Both DNP and dansyl presenting nanoallergens stimulated similar degranulation response to the hapten-BSA conjugated allergen at a 100- and 10-fold lower concentrations, respectively, demonstrating the higher potency of the platform (Figure 2(a) and (b)). Furthermore, any cross-reactions with liposomes without hapten-lipid conjugates (Blank) or cells primed with the other hapten-specific IgE was not detected. This confirmed nanoallergen specificity, and that the intensity of response was dependent on nanoallergen concentration.

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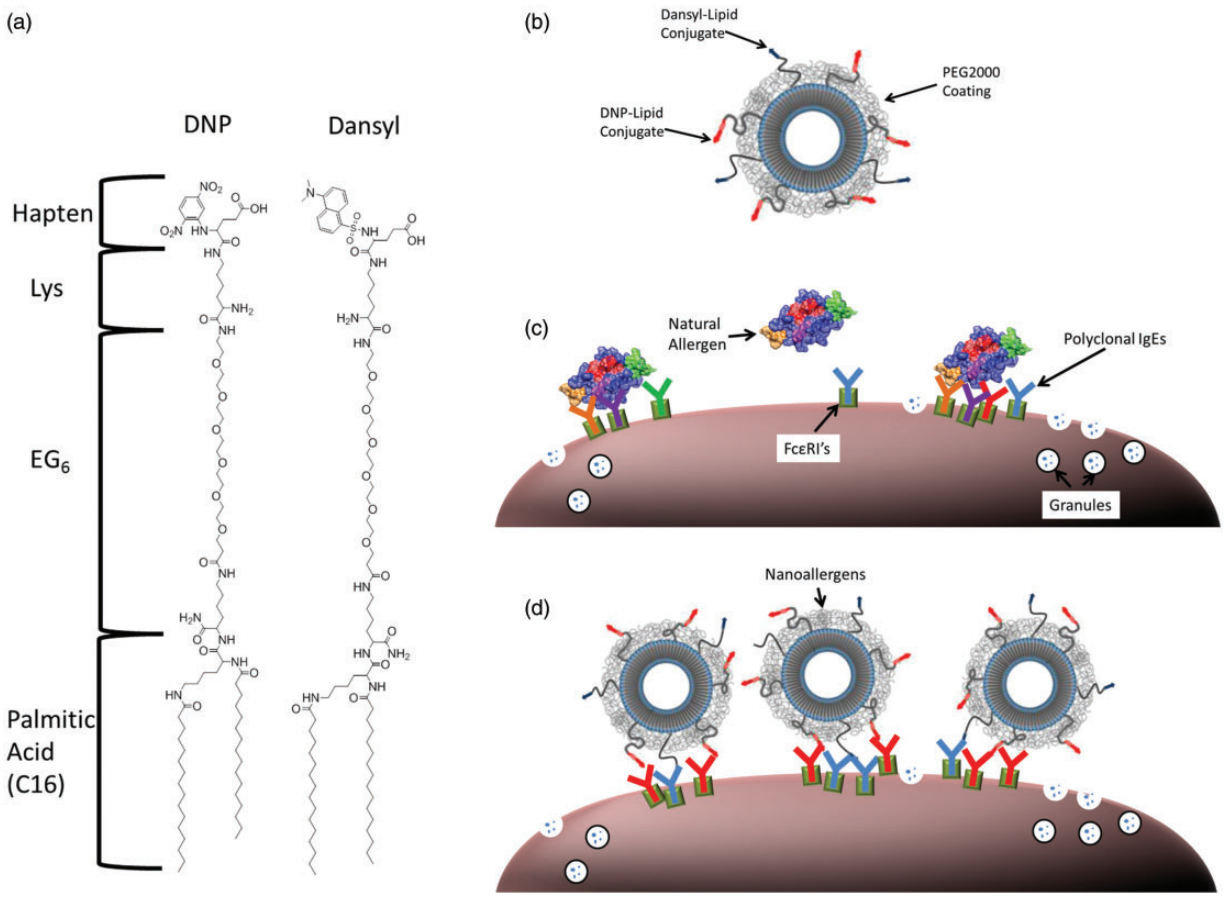

Figure 1 Nanoallergen design and formulation. (a) Chemical structures of hapten-lipid conjugates. (b) Schematic of nanoallegen liposome formulation demonstrating PEG2000 coating and haptens-lipid conjugates. Cartoons demonstrating mast cells (shown in gray) with primed FcεRI receptors with polyclonal IgE's responding to natural allergen protein (c) and mast cell degranulation induced through nanoallergen FcεRI receptor crosslinking (d). DNP: 2,4-dinitrophenol; IgEs: immunoglobulin E. (A color version of this figure is available in the online journal.)

The nanoallergens presented a similar response curve to hapten-BSA conjugates and common protein allergen molecules, indicating that they triggered the degranulation response in a similar manner. To confirm that the nanoallergens were binding specifically only to those RBL cells that present the corresponding IgEs on their surface prior to initiating degranulation, we performed flow cytometry experiments. Our results indicated that both dansyl and DNP nanoallergens demonstrated specific binding to RBL cells only primed with the analogous hapten-specific IgE (Figure 2(c)). The nanoallergens demonstrated a tapering of response at high concentrations. This was likely due to supraoptimal concentrations of the allergen causing excess IgE crosslinking and stimulating intracellular inhibitory pathways.[16] In order to confirm the activation of inhibitory pathways when the degranulation response plateaued, we concurrently performed a degranulation assay and a western blot with a nanoallergen loaded with both haptens, 5% DNP and 5% dansyl, to observe the expression of SHIP-1 protein during degranulation (Figure 2(d) and (e)). SHIP-1 was phosphorylated when a supraoptimal concentration of allergen caused overstimulation and activation of intracellular inhibitory pathways.[33] During the plateau of degranulation response, we demonstrated that SHIP-1 protein was phosphorylated, indicating the activation of intracellular inhibitory pathways.

Nanoallergen particle size and loading affects degranulation response

Particle size and peptide density can greatly affect the avidity a liposome has for the specified cell surface. We demonstrated that increasing particle size (50, 80, 100, and 200 nm diameter sizes were tested) while keeping other parameters (such as hapten loading) constant results in more potent degranulation responses (Figure 3(a) and (c)). Additionally, increasing the percent of loaded hapten-lipid conjugates (0.01, 0.1, 1, 2, 5, and 10% of total lipid) increased the maximum percent degranulation for similar reasons (Figure 3(b) and (d)). For the DNP nanoallergen, the percent loading did not have a significant effect on the degranulation; however, DNP nanoallergens demonstrated greater responses at lower concentrations with higher (10 and 5%) loading. The lower affinity dansyl nanoallergen demonstrated a drastic increase in degranulation response between 1 and 2% loading (Figure 3(d)). Overall, this data

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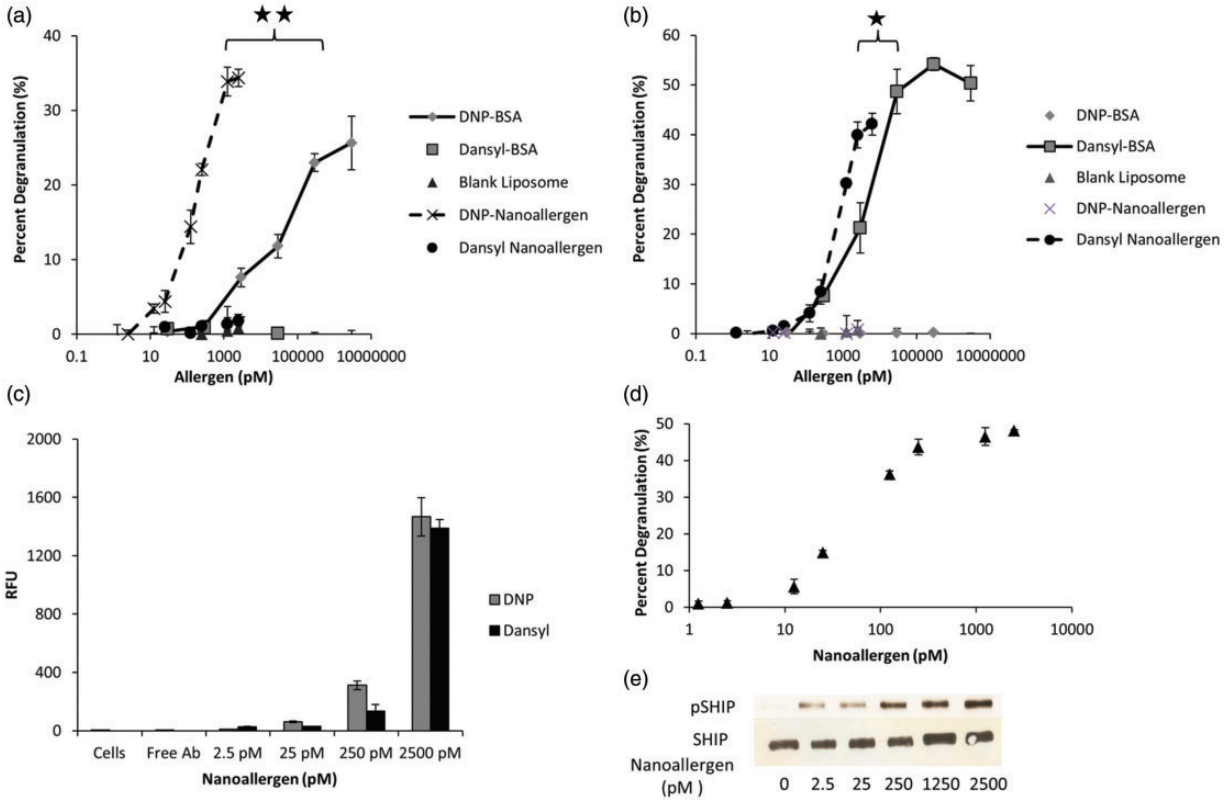

Figure 2 Nanoallergens stimulate degranulation in RBL-2H3 cells. RBL cells primed with 25% IgE^{DNP} (a) or IgE^{Dansyl} (b) and 75% orthogonal $IgE^{CyclinA}$ demonstrate degranulation responses similar to BSA-hapten conjugates. Single stars and double stars indicate a >10- and >100-fold decrease in allergen concentration to generate maximum response between BSA-hapten and nanoallergen. Flow cytometry (c) demonstrates specific binding to RBL cells primed with 50%/50% IgE^{DNP}/IgE^{Dansyl} to 2% hapten loaded nanoallergens at varying nanoallergen concentrations. (d) Cells were overstimulated with nanoallergens loaded with 5% DNP, 5% dansyl, and 50% cholesterol to achieve overstimulation. (e) Western blots demonstrating phosphylated SHIP-1 protein and total SHIP protein using the same stimulation conditions as the degranulation assay in part (d). RFU represents relative fluorescence units. DNP: 2,4-dinitrophenol; BSA: bovine serum albumin (A color version of this figure is available in the online journal.)

indicated the reliability of these single hapten loaded nanoallergens at inducing a strong degranulation response.

Nanoallergen binding and degranulation kinetics

To further demonstrate the utility of the nanoallergen platform, we performed a kinetic binding experiment. As demonstrated in Figure 2(d), the maximum nanoallergen binding to the cells did not relate to the maximum degranulation response. The data demonstrate that there is a threshold of binding necessary to stimulate maximum degranulation response, but any additional binding did not result in an increase in degranulation response. To address this question, we tested varying sizes of DNP nanoallergens using RBL cells primed with 25% DNP-specific IgE and observed their degranulation response at various time points between 2 and 120 min at 4°C (Figure 4). The data indicate that the number of bound particles increases between 1 and 20 min for the 100 and 200 nm particles, while the 50 nm particles steadily bound over 1 h. Additionally, the large liposomes had higher initial binding than the 50 nm liposome. We simultaneously observed degranulation from these same 50, 100, and 200 nm particles (Figure S–4). The degranulation experiments demonstrated an increasing degranulation response over 90 min for all particle sizes. However, the 50 nm particles triggered less degranulation at all time points, suggesting the decreased valency of the smaller particles reduced the degranulation response.

Hapten and IgE combinations affect degranulation response

The versatility of the nanoallergen platform is best exemplified when multiple types of haptens were loaded into the bilayer. Because protein allergens present multiple IgE binding epitopes on the same allergen, nanoallergens could readily emulate protein allergens through epitope heterogeneity and precise epitope loading. By loading both DNP and dansyl haptens on the same particle, we used nanoallergens to demonstrate the effects of antigen heterogeneity on degranulation response. We loaded nanoallergens with various ratios of DNP-lipid to dansyl-lipid while maintaining the total hapten-lipid loading at 2% of total lipid. Additionally, we varied epitope-specific IgE ratios when priming the RBL cells to simulate the variability in clinical IgE content (Figure S–5 (a) to (e), Table S–4). Figure 5 presents the EC_{50} values and maximum for each of these curves. The data demonstrate the complexity of the degranulation response and how many factors influence

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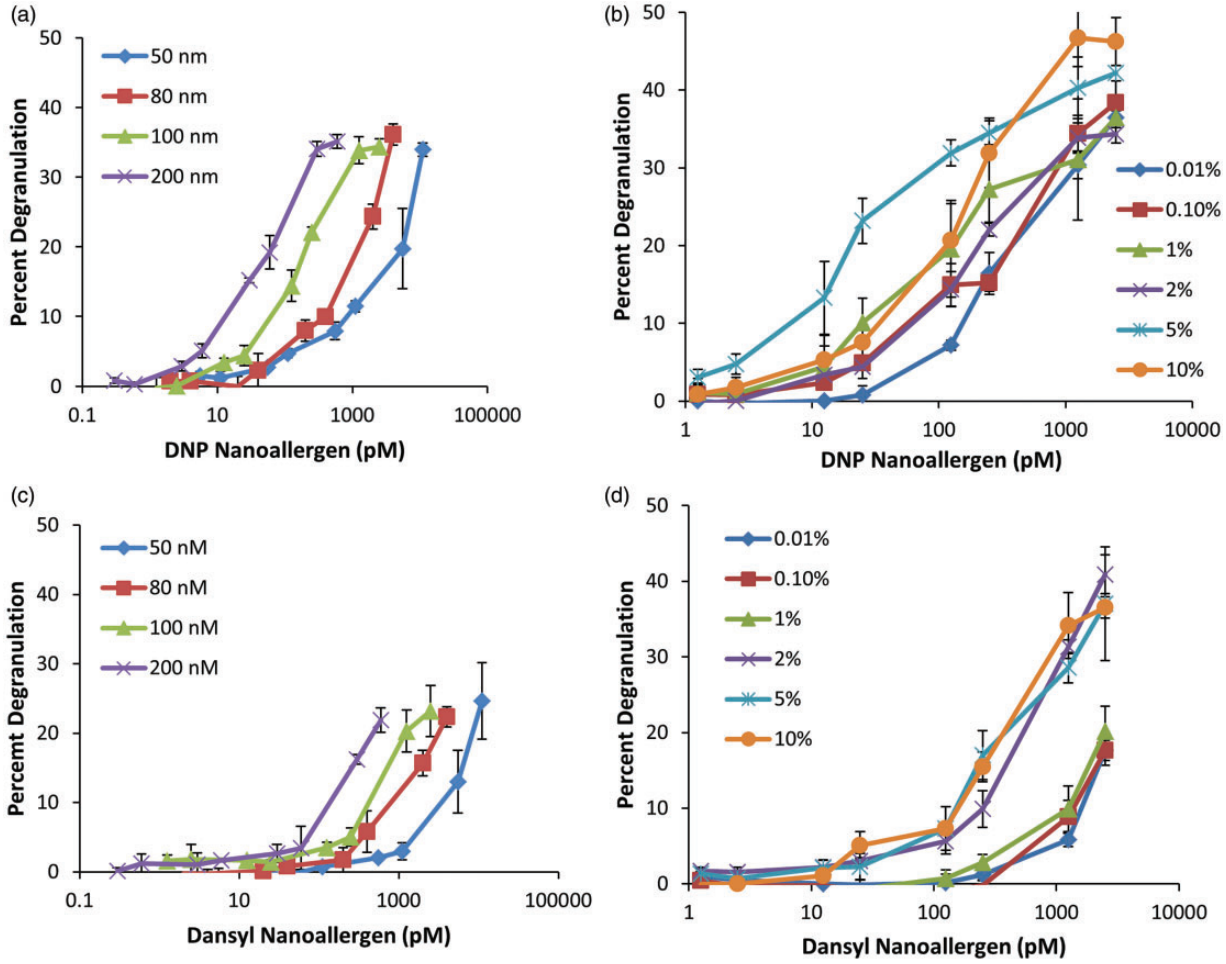

Figure 3 Nanoallergen formulations varying with particle size (50, 80, 100, and 200 nm) and hapten loading (0.01, 0.1, 1, 2, 5, and 10% of total lipid) affect degranulation response. Nanoallergen particle sizes alter the degranulation response for a DNP nanoallergen (a) and dansyl nanoallergen (c) at 2% hapten loading. Variation in degranulation response is seen in both DNP (b) and dansyl (d) nanoallergens when hapten loading is varied on a 100-nm particle. DNP: 2,4-dinitrophenol. (A color version of this figure is available in the online journal.)

degranulation response, such as particle valency, IgE ratios, and allergen concentration. Nevertheless, some trends were discernible and reliable. For example, the optimal degranulation response occurred for all lipid loading ratios at 12.5% IgE^{DNP} and 12.5% IgE^{dansyl}. Also, for most nanoallergens, there is a larger maximum response (30–40% of maximum) at low nanomolar nanoallergen, demonstrating nanoallergens ability to mimic protein allergen potency *in vitro*.

Discussion

The results presented in this paper establish the nanoallergen platform as a versatile and effective method for reliable and reproducible activation of cellular degranulation. The platform addresses several challenges of *in vitro* allergy models such as the difficulty of relating allergen binding affinity directly to a degranulation response given the complex nature of degranulation. Degranulation is affected by both allergen binding attributes such as size of IgE-FcεRI clusters and number of clusters as well as cellular properties such as downstream signal transduction. Here, we used the nanoallergen platform to systematically dissect and investigate aspects of allergen binding such as valency and monovalent affinity and observe their direct effects on degranulation responses using established *in vitro* degranulation assays.

By using hapten molecules with known affinities, we demonstrate the complexities of the allergen binding-degranulation relationship. As stated earlier, IgE^{DNP} and IgE^{dansyl} have different affinities for their respective haptens. Moreover, in an effort to widen their affinity difference, we conjugated both haptens to a glutamic acid residue (Figure S–1). The result was a nearly 10-fold difference in affinity, making the haptens a suitable pair for heterogeneous nanoallergens ($K_d^{DNP} = 15$ nM; $K_d^{dansyl} = 147$ nM, Table S–4, Figure S–6).[17] This difference in monovalent affinity translates to a stronger degranulation response for the higher affinity DNP nanoallergen than the dansyl nanoallergen (Table S–3). Likewise, increasing hapten loading, and therefore, valency, increases the degranulation response, although not in a linear fashion (Figure 3). For the dansyl nanoallergen, there was a large increase in response between 1% loading and 2% loading, but the

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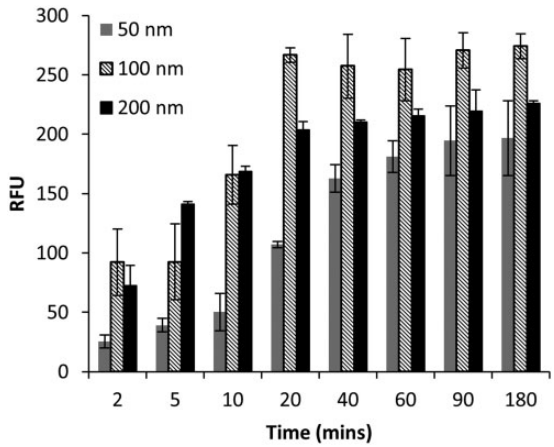

Figure 4 Kinetic binding of DNP nanoallergens. Nanoallergens of 50, 100, and 200 nm were loaded with 2% DNP-lipid and 600 molecules of fluorescent DiD dye per particle, incubated with RBL cells primed with IgE^{DNP} and then analyzed by flow cytometry at various time points. RFU: Relative Flourscence Units.

DNP nanoallergen did not have as clear a trend, instead only demonstrating marginally higher responses for higher hapten loading. This is likely due to the 10-fold difference in monovalent affinity between DNP–IgE^{DNP} and dansyl–IgE^{dansyl} interactions causing longer disassociation half-lives for a single DNP–IgE^{DNP} interaction. Degranulation requires the clustering of three or more IgE-FcεRI complexes, but given the high valency of nanoallergens and the rapid diffusion of IgE-FcεRI on the cell surface, it appears that one of the most crucial steps in a degranulation response is the binding of the second IgE-FcεRI complex.[7,35] Reduced dissociation kinetics from the cell surface increases the likelihood of a second IgE-FcεRI receptor diffusing to the nanoallergen and forming larger clusters. A weaker monovalent affinity would be reflected in a larger k_{off} for the dansyl-IgE^{dansyl}, resulting in a shorter disassociation half-life and increasing the likelihood of nanoallergen disassociating from the cell surface before a second IgE interaction can be formed. This suggests that there is a critical spacing distance between haptens which facilitates bivalent binding to the same IgE molecule, allowing nanoallergen–cell interactions to have increased half-lives, thereby increasing the likelihood of IgE-FcεRI cluster formation. This bivalent IgE binding is a plausible explanation for the increase in degranulation response between 1 and 2% for dansyl nanoallergens (Figure 3(d), Table S–2).

Another important factor that impacts degranulation response was epitope heterogeneity. The higher affinity (DNP) hapten caused the strongest response at lower concentrations when it was the only epitope present, due likely to its high-monovalent affinity (Figure 5). Nevertheless, when nanoallergens were prepared presenting both DNP as well as dansyl haptens at varying ratios, intensity of cell response varied at different concentrations. For example, the EC_{50} value for the 12.5/12.5/75% $IgE^{DNP}/IgE^{dansyl}/IgE^{dansyl}$ ratio increased from 310 ± 80 pM with the 2% loaded dansyl nanoallergen to 600 ± 50 pM for the 0.1/1.9% dansyl/DNP hapten loaded nanoallergens, demonstrating a p value of less than 0.01 (Figures 5(a) and S–5). Even though a higher affinity hapten (DNP) was added to the nanoallergen, the data demonstrate a decrease in degranulation response. We predict the difference originating from the 2% loading density providing the critical distance for hapten separation that can accommodate bivalent binding of dansyl haptens to a single IgE^{dansyl} (Table S–2). The incorporation of DNP hapten into the nanoallergens results in a slight decrease in the density of dansyl on the particle surface and decreasing the statistical likelihood of an IgE^{dansyl} molecule from binding bivalently to a nanoallergen, resulting in a reduction in the avidity of the nanoallergen (Figure S–7). Nevertheless, for nanoallergens of greater than 1% DNP loading, this reduction in bivalent binding was offset by the increased monovalent affinity of DNP and resulted in enhanced degranulation responses. For example, the EC_{50} value for 12.5/12.5/75% $IgE^{DNP}/IgE^{dansyl}/IgE^{dansyl}$ ratio decreased as the DNP hapten ratio was increased from 0.1 to 1% ($p < 0.05$, Figures 5(a) and S–5). Additionally, this effect is seen when reversing the two haptens; as 0.1% dansyl is introduced into a DNP nanoallergen, a statically significant ($p < 0.01$) increase in EC_{50} value occurs at every IgE ratio except 25 and 24% IgE^{dansyl} (Figure 5(a)). While a small decrease in affinity would be expected due to the introduction of a lower affinity hapten, such large EC_{50} value differences suggest that the introduction of dansyl haptens on the surface is also preventing bivalent IgE^{DNP} binding.

The nanoallergen studies presented here also reveal several more nuanced aspects of a degranulation response. The higher the overall nanoallergen valency, the more IgE–hapten interactions can be formed and the stronger the degranulation response (Table S–2). Additionally, avidity was not the only factor determining the potency of nanoallergens. The non-linear relationship between hapten surface density and the degranulation response demonstrated that there was an optimal avidity for the intensity of the maximum degranulation response for a given nanoallergen formulation, and this optimal response was also mediated through intracellular inhibitory cascades. Our data indicate that the maximum degranulation response did not occur when the highest number of nanoallergens was bound on the cell surface. As demonstrated in Figure 2, nanoallergen binding was highest at 2.5 nM for 2% loaded DNP nanoallergens, while the maximum degranulation response occurred at 250 pM when these same nanoallergens were used to stimulate degranulation (Figure S–5). A possible explanation for this is the presence of intracellular inhibitory cascades that activate during overstimulation.[36] Furthermore, as demonstrated by Figure 2(d) and (e), nanoallergens at supraoptimal concentrations stimulated intracellular inhibitory cascades (SHIP protein).

Studies conducted using nanoallergens have the potential to reveal detailed and critical information about allergen proteins and their epitopes that even the purified natural allergen proteins themselves cannot deliver. Factors such as affinity, valency, epitope heterogeneity, and intracellular inhibitory cascades are crucial complicating factors for degranulation responses. Nanoallergens allow for precise

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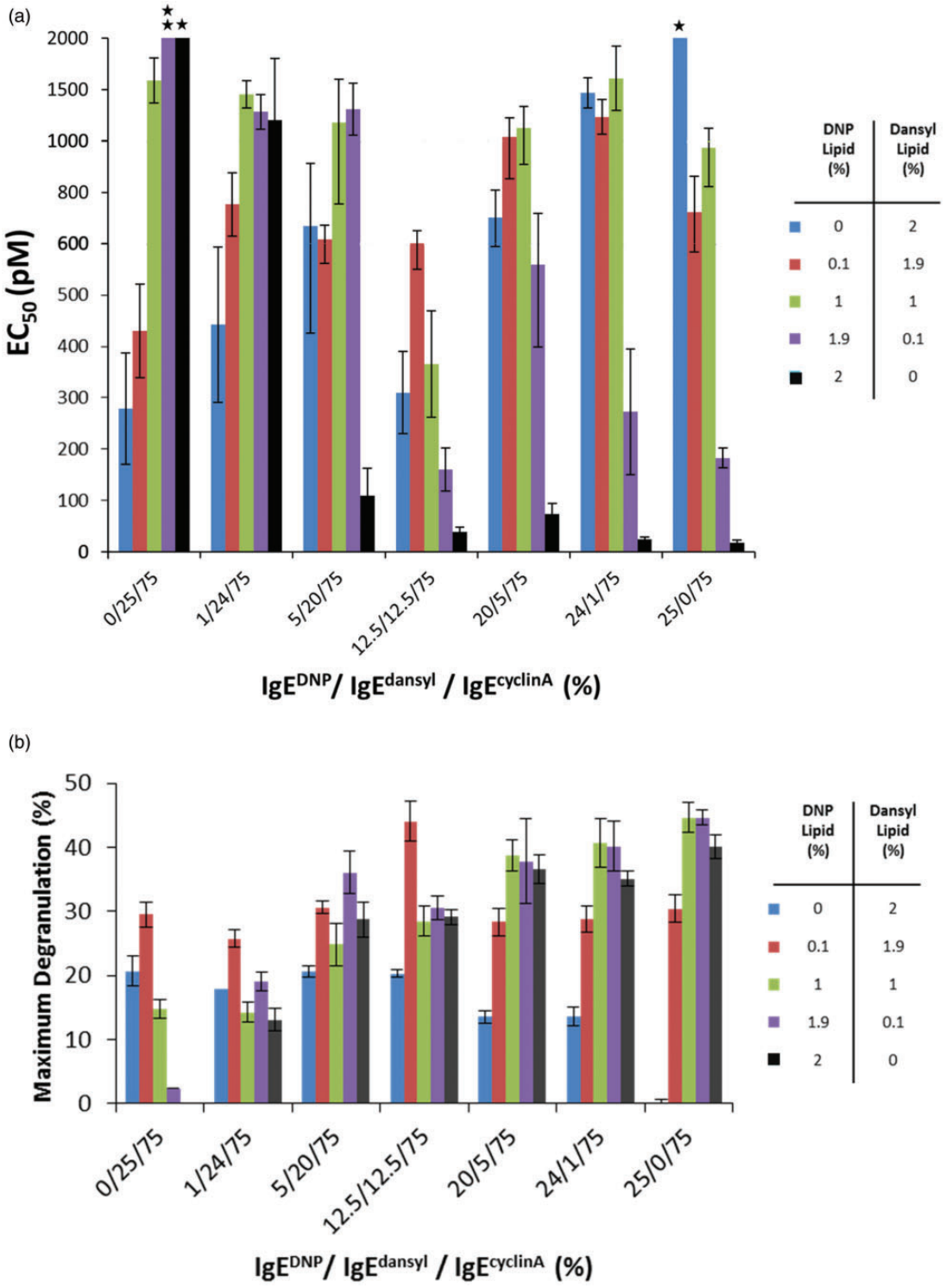

Figure 5 EC_{50} and maximum degranulation values for DNP-dansyl combination nanoallergen. The IgE ratios used to prime the RBL-2H3 cells were varied as demonstrated on the *X* axis. Each color bar graph corresponds to hapten-lipid loading ratios. *Note*: Stars indicate a lack of response at all concentrations tested. Double stars indicate EC_{50} values above 5000 pM. EC_{50} values are in (a) and maximum degranulation values are shown in (b). DNP: 2,4-dinitrophenol; IgE: immunoglobulin E. (A color version of this figure is available in the online journal.)

control over affinity, valency and provide immunogenic data on individual epitopes. Meanwhile, without sophisticated binding experiments, it is difficult to decipher which epitopes on allergen proteins are binding and the kinetics of these interactions. Finally, allergen proteins can only have their specific epitopes be altered with site specific directed mutagenesis, which is rather time consuming and challenging, making screening for important allergy epitopes very difficult. In addition, nanoallergens can be used to assess the kinetics of allergen binding (Figure 4) due to their ability

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to readily incorporate fluorescent dyes. Likewise, nanoallergens offer method to assess the influence on parameters such as allergen size on degranulation kinetics (Figure S–4). Although liposomes are not as stable as allergen proteins and require more sophisticated synthesis techniques, the benefits of nanoallergens outweigh the drawbacks.

In conclusion, the nanoallergen platform presented in this paper provides an efficient and versatile platform for allergy research. Nanoallergens offer several advantages over the current BSA-hapten system. The ability to generate very nanoallergens of very high valency allows them to trigger degranulation responses at similarly low (nanomolar to picomolar) concentrations as native allergen proteins. For example, peanut allergens, Ara h2 and Ara h6, have been identified to have EC_{50} values in cellular studies in the low picomolar range using various sera from highly allergic individuals, and these potent responses are not possible to emulate with linear peptide epitopes without a highly multivalent platform such as nanoallergens.[37] Even with the high-affinity DNP hapten, DNP-BSA with a valency of 18 that was used in this study as a control was only able to trigger degranulation in the nanomolar range ($EC_{50} = 26 \pm 9$ nM) while a 2% loaded DNP nanoallergen (valency of 800) was able to trigger degranulation in the picomolar range ($EC_{50} = 180 \pm 20$ pM) (Figure 2). In addition to the increase of valency, nanoallergens can also take advantage of simultaneous bivalent interactions on a single IgE antibody and further intensify the degranulation response. The atypical size and capacity to display epitopes at a very high valency make nanoallergens a very viable platform for studying and sufficiently ranking peptide epitopes with low affinities. Furthermore, nanoallergens offer precise control over allergen valency, which in turn can be used to study the kinetics of IgE-FcεRI clustering. These particles can be easily tagged with fluorescent molecules, facilitating studies of IgE-FcεRI cluster size and shape on immunogenicity. Finally and most importantly, nanoallergens can be formulated with epitope heterogeneity and precise control over epitope ratios on the particles. For this study, only two hapten molecules were used, but nanoallergens can readily display any number of hapten molecules or epitope peptides in any combination. This means nanoallergens can easily be formulated to emulate various immunogenic proteins simply by changing the epitope loading ratios. This process could also be used to select epitopes most crucial in stimulating degranulation and provide critical information for future degranulation inhibitor designs.

Author contributions: PED planned and conducted experiments, analyzed data, and wrote the text of the paper. MRV and VJP contributed to the experiments and data analysis for Figure 5. TK provided technical assistance and contributed to paper editing. BB provided technical guidance, supervised the study, and edited the paper.

ACKNOWLEDGEMENTS

We thank Dr. Bill Boggess at the Mass Spectrometry and Proteomics Facility in the University of Notre Dame for the use of MS instrumentation and the Center for Environmental Science and Technology for the use of their DLS instrument. This work was supported by the NIH grant number R01AI108884.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Supplementary Information for Nanoallergens: A Multivalent Platform for Studying and Evaluating Potency of Allergen Epitopes in Cellular Degranulation

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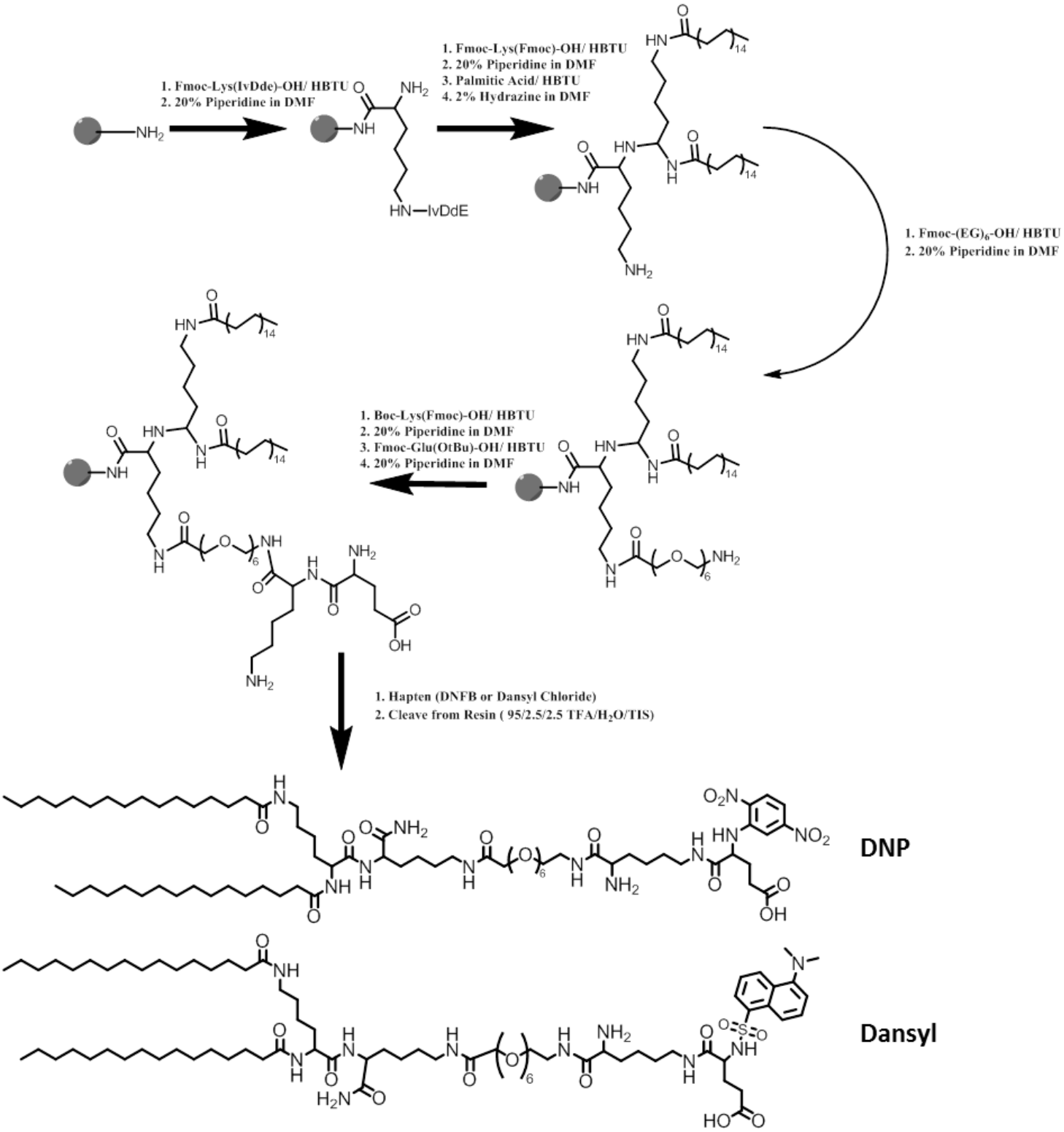
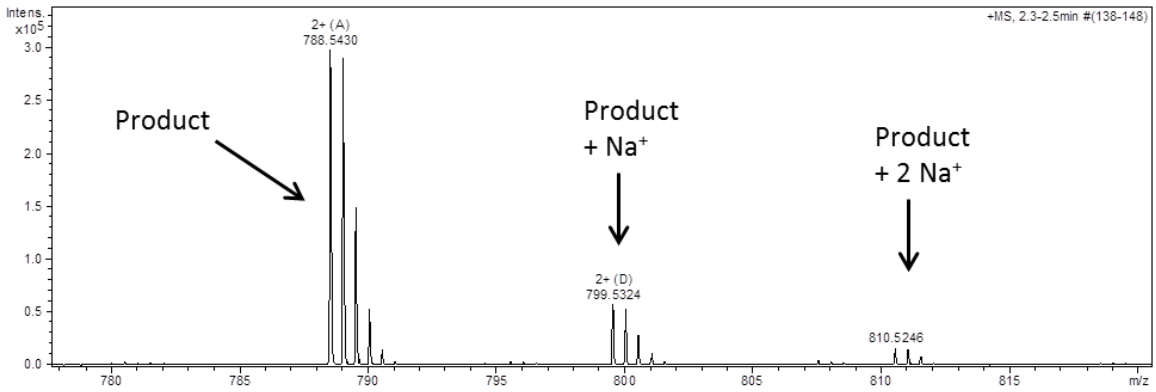


Figure S-1. Synthetic Scheme of Hapten-Lipid conjugates. DNP and Dansyl haptens were conjugated using Fmoc chemistry on a solid support using the scheme above.

Table S-1. Hapten-lipid variations. Listed here are the respective hapten-lipids molecules, the amino acid reside conjugated to the hapten, the monovalent affinity of the hapten-amino acid conjugate to its respective IgE, and the EC_{50} value and maximum degranulation of a 2% loaded hapten-lipid nanoallergen. Degranulation experiments were performed using RBL-2H3 cells primed with 25 % specific hapten IgE and 25% orthogonal IgE. Stars (*) indicate data from manuscript by Handlogten *et al.*

Hapten	Reside	K_d (nM)	EC_{50} (pM)	Max (%)
DNP	Glu	15 ± 2.5*	10.7 ± 2	28.1 ± 0.6
	Lys	105 ± 15*	45.4 ± 4	24.6 ± 0.6
Dansyl	Glu	147 ± 45	279 ± 108	20.7 ±2.4
	Lys	23 ± 12	23 ± 13	33.3 ± 3

A.



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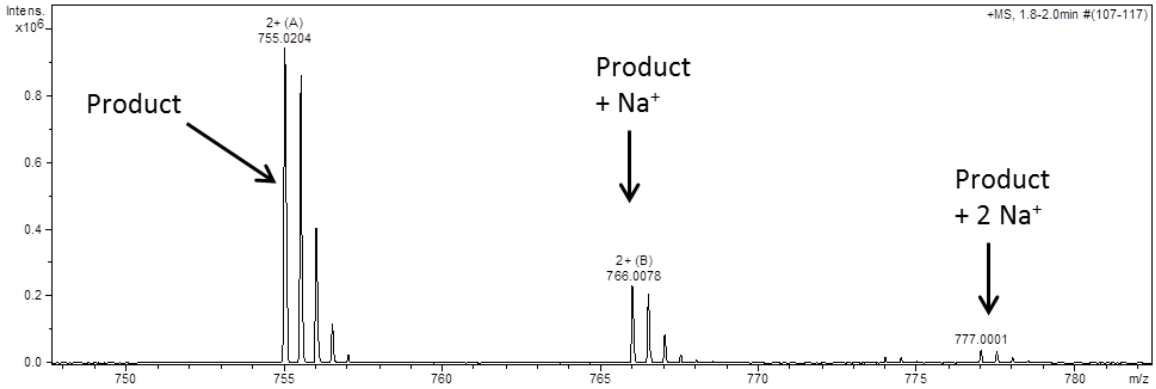


Figure S-2 Mass Spectrometry analysis of Lipid-Hapten conjugates. Lipid-Dansyl product had an expected mass of 1575.06 Da and was seen in A.) with a doubly charged peak with a m/z of 1575.08. Likewise, the Lipid-DNP conjugate had an expected mass of 1508.01 Da, demonstrated in B.) with a doubly charged peak corresponding to a m/z of 1508.04. Both products also had two sodium adducts.

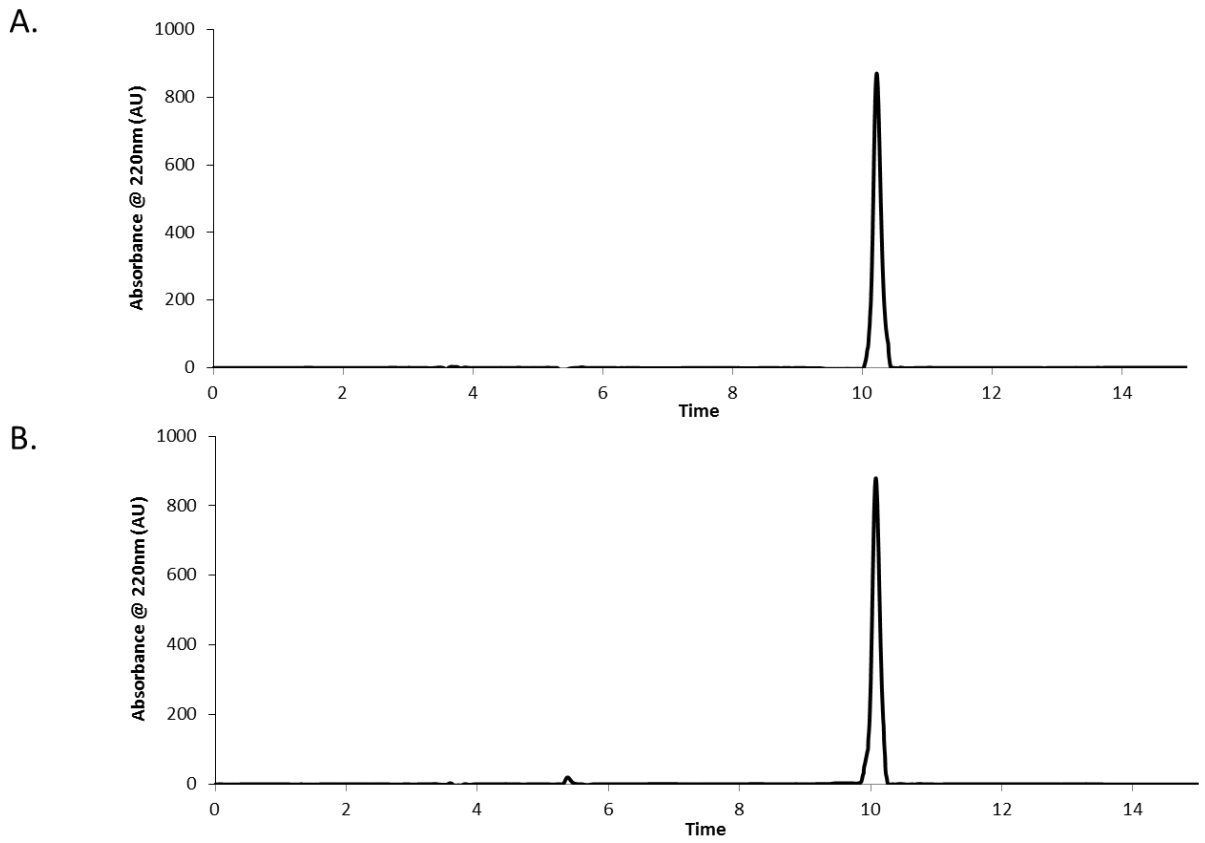


Figure S-3 RP-HPLC Purity Analysis. Both molecules demonstrated >95% purity by peak area using RP-HPLC described in the methods section. The lipid-DNP conjugate (A) and lipid-dansyl (B) conjugate both showed a single peak at 220nm at around 10 minutes.

Table S-2. Homologous particle characterization. Liposomal area was estimated assuming a sphere and the total number of lipids calculated. The hapten spacing was calculated from the hapten density assuming the haptens are evenly spaced on a hexagonal grid.

Diameter	DNP-Lipid	Dansyl-Lipid	Liposome Area	Number of Lipid/Liposome	Total Haptens (Est*)	Hapten Density	Hapten Spacing	EC_{50}
nm	(%)	(%)	nm2			(nm^2/ hapten)	(nm)	pM
50	2	0	7850	18142.5	181.4	43.3	8.2	2790±670
80	2	0	20096	50002.5	500.0	40.2	7.9	1000±138
100	2	0	31400	80092.5	800.9	39.2	7.8	10.9±2
200	2	0	125600	336742.5	3367.4	37.3	7.6	59.9±11.9
50	0	2	7850	18142.5	181.4	43.3	8.2	5580±380
80	0	2	20096	50002.5	500.0	40.2	7.9	1140±98
100	0	2	31400	80092.5	800.9	39.2	7.8	279±108
200	0	2	125600	336742.5	3367.4	37.3	7.6	337±115
100	0.01	0	31400	80092.5	4.0	7840.9	109.9	354±70.1
100	0.1	0	31400	80092.5	40.0	784.1	34.7	267±40
100	1	0	31400	80092.5	400.5	78.4	11.0	222±41.4
100	5	0	31400	80092.5	2002.3	15.7	4.9	27.4±5.24
100	10	0	31400	80092.5	4004.6	7.8	3.5	162±25.8
100	0	0.01	31400	80092.5	4.0	7840.9	109.9	2490±78.3
100	0	0.1	31400	80092.5	40.0	784.1	34.7	1250±27.7
100	0	1	31400	80092.5	400.5	78.4	11.0	1140±110
100	0	5	31400	80092.5	2002.3	15.7	4.9	324±42
100	0	10	31400	80092.5	4004.6	7.8	3.5	354±35.4

Table S-3. Nanoallergen Particle Sizing. The particle sizes of 2% loaded DNP and Dansyl nanoallergens were determined using DLS analysis. Different pore sizes on extrusion filters created particles of varying size.

Liposome	Average Diameter (nm) (2% DNP)	Average Diameter (nm) (2% Dansyl)
50 nm	57.1 ± 0.2	57.4 ± 0.2
80 nm	89.3 ±2.8	88.4 ±1.1
100 nm	110.1±1.4	113.5±1.2
200 nm	180.5±1.1	175.8±0.7

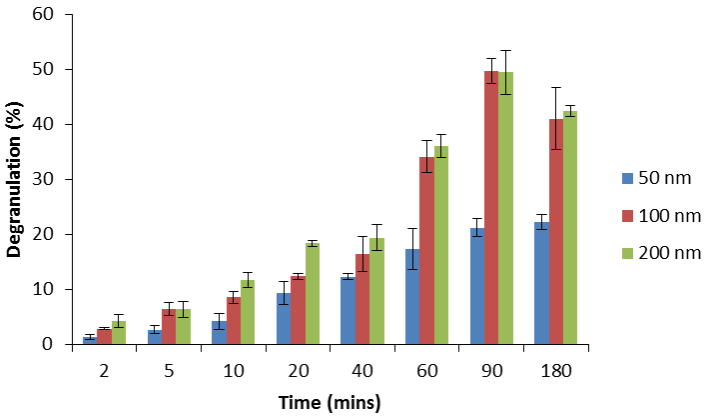
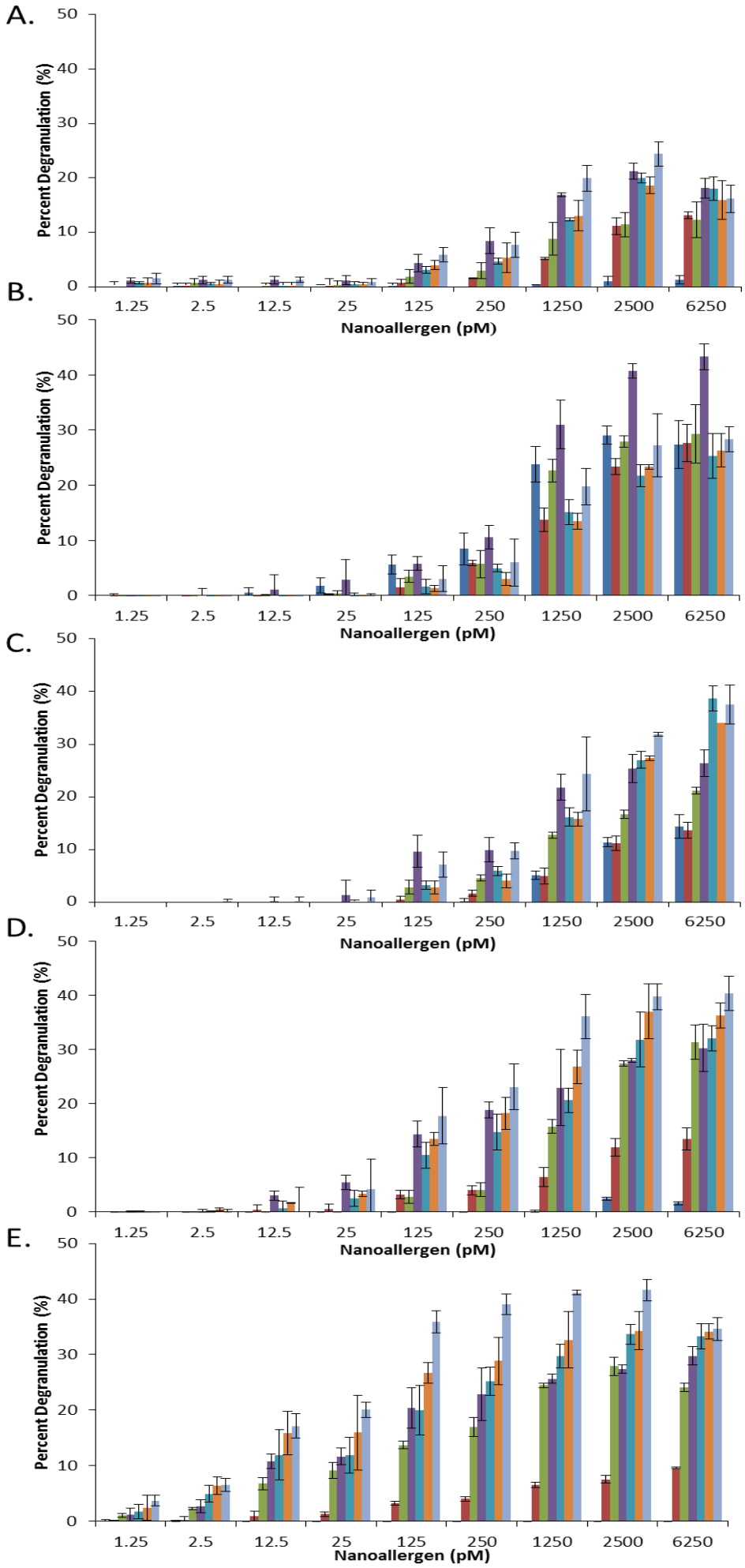


Figure S-4. Nanoallergen degranulation increases over time. RBL cells primed with IgE^{DNP} demonstrate increasing responses to 2% loaded DNP nanoallergens over the course of an hour.



IgE^{DNP} (%)	IgE^{Dansyl} (%)	$IgE^{CyclinA}$ (%)
0	25	75
1	24	75
5	20	75
12.5	12.5	75
20	5	75
24	1	75
25	0	75

Figure S-5. Hapten and IgE combinations demonstrate varying degranulation response. 100 nm nanoallergens with 2% hapten loading were incubated with cells primed with 75% orthogonal $IgE^{CyclinA}$ and between 0-25% IgE^{DNP} with IgE^{Dansyl} comprising the remainder. Liposomes consisted of 0% DNP (A.), 0.1% DNP (B.) 1% DNP (C.), 1.9 % DNP (D.) and 2% DNP (E.) with the remainder Dansyl for a total of 2% by mole hapten loading.

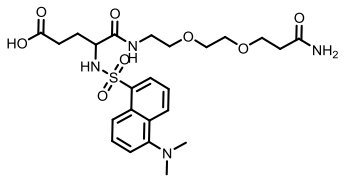
Table S-4. EC_{50} values and maximums of heterogeneous Nanoallergens.

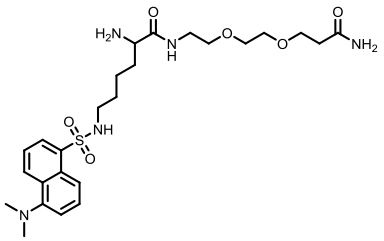
			Percent Lipid-Hapten Loading (DNP/Dansyl)									
	Antibody Ratio		0/2		0.1/0.9		1/1		1.9/0.1		2/0	
Cyclin A	DNP	Dansyl	EC_{50}	Max	EC_{50}	Max	EC_{50}	Max	EC_{50}	Max	EC_{50}	Max
75	0	25	280±100	20.7±2.38	430±90	29.5±1.9	1600±200	14.8±1.5	>6250	N/A	>6250	N/A
75	1	24	440±150	18.0±1.9	750±120	25.7±1.4	1450±130	14.3±1.5	1280±170	19±1.4	1200±600	13.1±1.7
75	5	20	670±200	20.6±2.6	620±55	30.6±0.9	1180±420	24.8±3.3	1300±250	36±3.3	110±53	28.7±2.7
75	12.5	12.5	310±80	20.3±1.9	600±50	44.1±3.0	365±100	28.5±2.3	160±42	30.5±1.9	39.5±8.4	29.1±1.2
75	20	5	700±100	11.7±1.1	1040±190	28.3±2.1	1120±210	39.8±2.4	560±160	37.8±6.6	74.0±20	36.6±2.3
75	24	1	1470±150	11.5±0.6	1230±170	28.9±2.1	1600±310	40.8±3.8	270±120	40.2±3.9	24.5±4.7	35.6±1.2
75	25	0	>6250	N/A	720±140	30.5±2.1	970±150	44.7±2.3	180±19	41.1±1.2	18.4±4.2	40.1±1.8

Glu-Dansyl

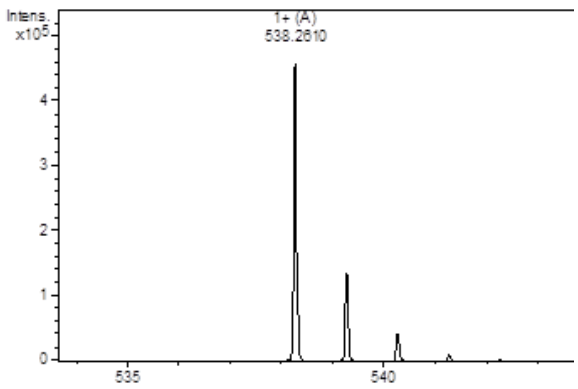
Lys-Dansyl

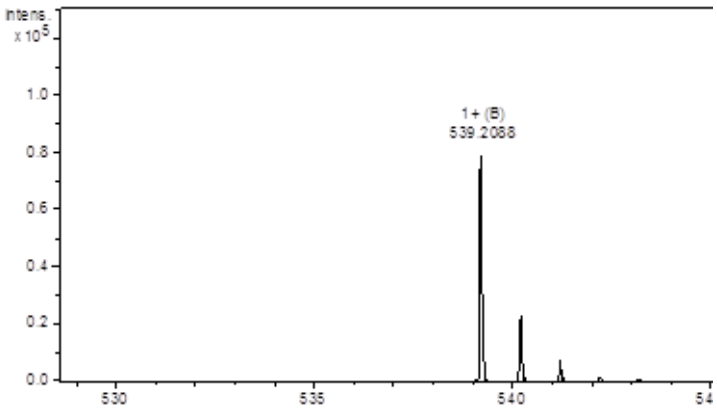
A.



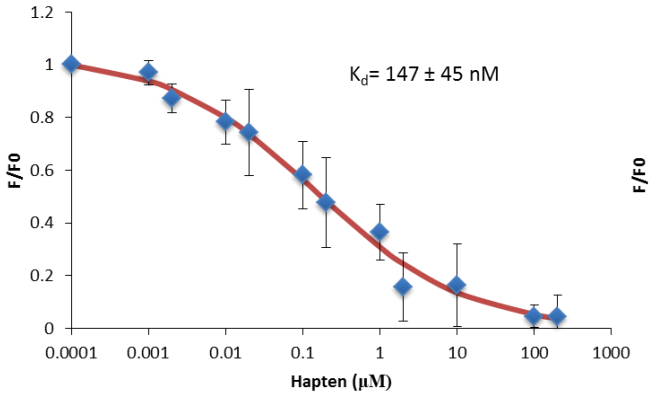


B.





C.



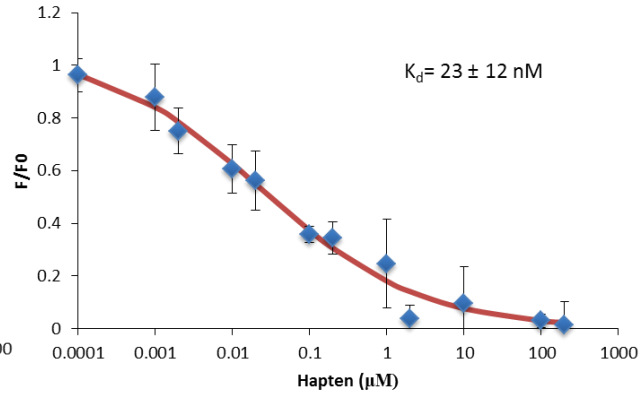


Figure S-6 Dansyl amino acid conjugates. Two different amino acids were conjugated to dansyl using Fmoc solid phase peptide synthesis. Their structures are shown in A. and their molecular weights were confirmed with high resolution mass spectroscopy (B.) A fluorescence quenching titration was then performed with the compounds and IgE^{dansyl} and a disassociation constant (K_d) was calculated (C.).

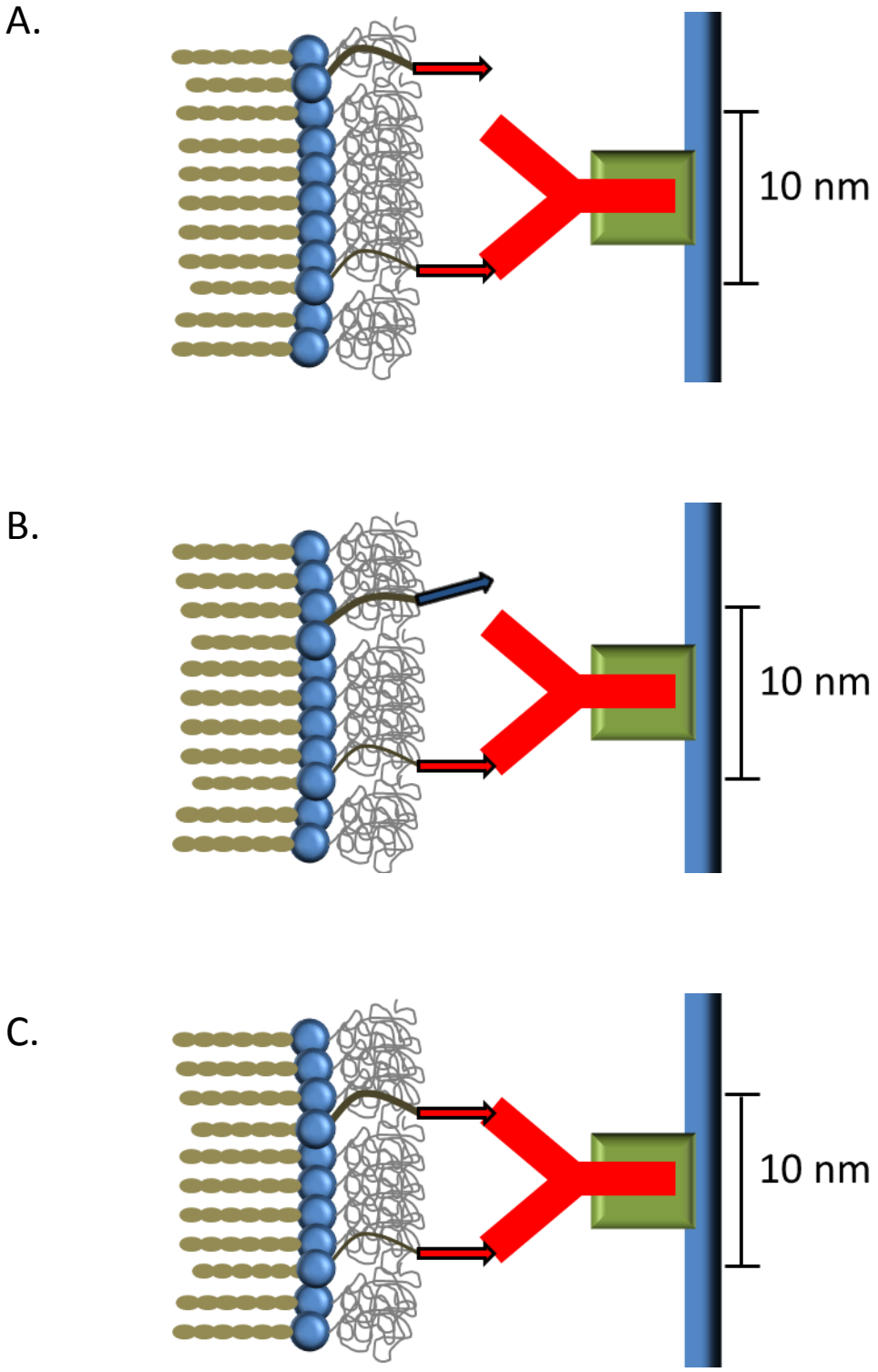


Figure S-7. Schematic demonstrating the divalent avidity enhancement on a single IgE. When the hapten molecules are spaced too far apart, divalent interactions on the same IgE molecule are less likely during a shorter timescale (t< 20 mins) as seen in A. Likewise, divalent interactions are not likely when the haptens are heterologous (B). Rapid, divalent interactions that would likely improve the degranulation response can occur on the same IgE with the proper hapten spacing under 10 nm as seen in C.