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Attaching histidine-tagged peptides and proteins to lipid-based carriers through use of metal-ion-chelating lipids

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Abstract

The therapeutic potential of selected peptides and proteins is enormous, with applications ranging from use as therapeutic vaccines, as modulators of intracellular signaling pathways and as highly selective agents capable of recognizing unique extracellular targets. We have been pursuing development of hybrid lipid-based carrier formulations designed to take advantage of the therapeutic benefits of peptides selected for their ability to act in a complementary fashion with the carrier system. In this regard, it is critical to have simple and versatile methods to promote and control the binding of diverse peptides to a broad range of carrier formulations. As demonstrated here, recombinant proteins and synthetic peptides containing poly-histidine residues (4 to 10) can be specifically bound to liposomes containing a metal-ion-chelating lipid, DOGS-NTA-Ni. The potential of this approach is demonstrated using two functional peptides, AntpHD-Cw3 (applications for vaccine production) and AHNP (specificity for Her-2 expressing cells).

Keywords: Metal-ion-chelating lipid; Liposome; Peptide; Poly-histidine tag; Targeting; Delivery

1. Introduction

Hybrid delivery systems contain two or more components; each selected to achieve a form of targeted delivery but combined into a single formulation. Importantly, neither delivery system alone can achieve what the combined formulation can. This is best illustrated in our recent studies with AntpHD-Cw3 recombinant peptide. This peptide comprises the homeodomain of *Antennapedia* (AntpHD), a protein which is able to spontaneously cross cell membranes to deliver a cytotoxic T lymphocyte (CTL) epitope, Cw3 to the MHC class I presentation pathway and to prime cytotoxic T cells [1,2]. This protein delivery system, on its own, has proven to be of little value in vivo because of sensitivity to protease degradation and inefficient access to the antigen presenting cells (APCs) of the immune system. We proposed and demonstrated that a liposomal formulation of the AntpHD-Cw3 recombinant peptide would overcome these difficulties by protecting the peptide and by delivering the peptide more efficiently to the appropriate APCs. In brief, the liposomal delivery system was used to deliver a peptide delivery system, and the combined delivery system has been termed a hybrid formulation (Fig. 1).

Having considered this achievement, one can envision a number of variations to this novel strategy. One recognized variant would include the use of therapeutically active antibodies attached to a liposomal drug formulation. The potential to use liposomes as carriers for delivering pharmaceutically active agents is well recognized [3,4] and the

Abbreviations: DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; DSPE-PEG$_{2000}$, 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*n*-[poly(ethylene glycol)$_{2000}$]; Chol, cholesterol; DOGS-NTA-Ni, 1,2-dioleoyl-*sn*-glycero-3-{[*n*(5-amino-1-carboxypentyl)iminodiacetic acid]succinyl} (Nickel Salt); AntpHD-Cw3, *Antennapedia* homeodomain fused to Cw3 epitope; ME, mercaptoethanol; RT, room temperature (22 °C); AHNP, peptidomimetic of Herceptin (FCDGFYACYMDV); APC, antigen presenting cell; CTL, cytotoxic T lymphocyte

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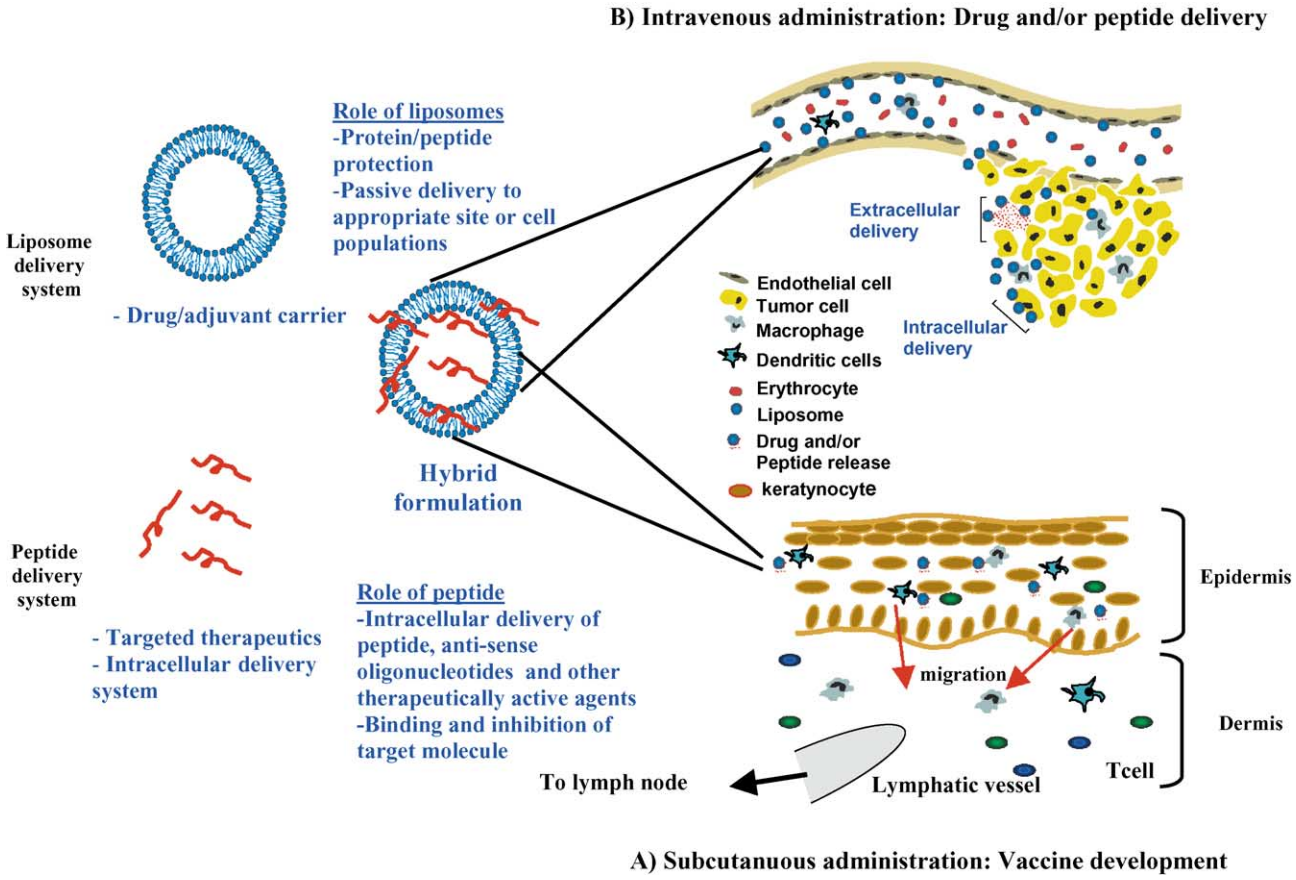

Fig. 1. Design of hybrid delivery systems using liposomes and peptides. The distinct roles of liposomes and peptides can be combined for various applications including vaccine development (A) and targeted drug delivery (B).

goal of targeting liposome through use of monoclonal antibodies to achieve improved selectivity and delivery of drugs to defined cell populations is still being actively pursued [5]. However, recent successes with monoclonal antibodies exhibiting therapeutic activity when administered intravenously [6], suggest the possibility of using these antibodies in combination with lipid-based carriers to prepare hybrid delivery systems as opposed to a targeted formulation. This may be viewed more as a conceptual distinction; however, the design features being pursued would be quite unique. A practical application of this idea would include development of Herceptin [7] or Rituximab [8] attached to a liposomal anticancer drug in a manner that could improve the therapeutic activity of the antibody as well as the liposomal drug. A rationale for Herceptin/liposomal doxorubicin has been proposed by our research team based on the known synergistic action of Herceptin and doxorubicin when combined to treat breast cancer [9]. Although clinically promising, the combination of Herceptin and doxorubicin engenders a significant increase in cardiac toxicity. A hybrid formulation comprising Herceptin with liposomal doxorubicin should enhance the delivery of the antibody to the disease site population and, provided its bioavailability and activity have not been compromised, the combination of liposomal doxorubicin and Herceptin should be enhanced compared to either agent alone or administered as an unlinked combination. The latter approach should be considered as distinct from the tactics of others who have defined methods for the covalent attachment of proteins to liposomes with the specific aim of targeting the liposomes to a defined cell population. This approach has been elegantly demonstrated by Park et al. [30] for liposomes with anti-Her-2 antibodies covalently coupled to liposomes.

Several therapeutically active peptides have been described and we have considered a few of these for development of the hybrid formulations described here. These include RGD peptides, the integrin–ligand interactions inhibitors [10] that can be used to probe integrin functions in various biological systems; the AHNP peptide derived from the anti-p185$^{Her\text{-}2/neu}$ antibody (Herceptin) [11], shown to be effective in limiting tumor growth in vivo; and AntpHD peptide vector demonstrated to be able to deliver CTL epitope to APC and induce CTL responses for vaccine development [2].

Our enthusiasm for the potential of hybrid protein/lipid delivery systems has led us to reconsider standard methods of combining peptides with lipid-based delivery systems. In particular, covalent and non-covalent means of attaching

peptides to lipid structures have proven to be reasonably inefficient and the methods must be customized to the protein/peptide of interest [12,13]. This is a time-consuming and frustrating exercise that is often difficult to consider when working with materials that are available in small quantities and are expensive to produce. Our experience with synthetic peptides and recombinant proteins, however, led us to an interesting solution that could provide versatility and efficiency in preparing hybrid carriers as well as offering the potential to easily control binding and dissociation parameters.

It is well established that electron-rich ligands such as histidine, tryptophane or cysteine bind with relatively high affinity to electropositive transition metals, including Co^{2+}, Ni^{2+}, Cu^{2+} and Zn^{2+}. This observation has been exploited to allow the selective purification of recombinant proteins that have been engineered to contain a stretch of His residues, typically at the N- or C-terminus [1,14], a method commonly referred to as immobilized metal affinity chromatography (IMAC). The presence of 4 to 10 contiguous histidine residues provides a tag that can stably bind a metal chelate affinity column and withstand extensive washing to remove non-specifically bound proteins. Recently, metal-ion-chelating lipids have been synthesized and used to study proteins, prepared with a His-tag, immobilized on a lipid monolayer. These previous studies were designed to determine structural information on diverse proteins including membrane proteins and to study the interactions of enzymes with their ligands by crystallography [15–17]. The present manuscript describes a new technology to improve and control the association of peptides to liposomes that exploits the affinity between histidine-tagged peptides and liposomes containing metal-chelating lipids. In particular, two peptides, (His)10-AntpHD-Cw3 and (His)4-AHNP were used to demonstrate rapid and efficient attachment of the peptides to liposomes. Importantly, it has also been shown that both peptides conserved their biological functions.

2. Results and discussion

2.1. AntpHD-Cw3-liposome hybrid prepared using liposome incorporated DOGS-NTA-Ni lipid

AntpHD-Cw3, a 128-amino acid peptide was prepared with a 10-histidine sequence tag at its amino terminal end. We have previously shown that incorporation of this peptide into liposomes (DOPC/Chol) achieved less than 50% association efficiency and this was reduced to 35% when those liposomes had surface-associated polyethylene glycol. The incorporation of this recombinant peptide is due primarily to its hydrophobicity, an attribute that is linked to its ability to spontaneously cross plasma membranes [1]. The studies illustrated in Fig. 2 were designed to determine whether the incorporation efficiency of this recombinant peptide could be improved when 10 mol% of DOGS-NTA-Ni lipid was incorporated into DOPC/Chol liposomes. This liposome was also prepared with 5 mol% of DSPE-PEG_{2000}, a lipid that inhibits non-specific association of the peptide with the liposomes. The elution profile shown in panel A clearly indicates that close to 100% of the added His-tagged protein could be associated with these liposomes, and peptide-to-lipid ratios of 20 μg/μmol were obtained (Fig. 2A). In the absence of the nickel-chelated lipid (Fig. 2B), peptide-to-lipid ratios of 6 μg/μmol were obtained, a result that is consistent with our previous data demonstrating spontaneous association of AntpHD-Cw3 with liposomes. Although not shown here, we established that 10 mol% DOGS-NTA-Ni provided optimal AntpHD-Cw3 binding attributes (recombinant peptide binding in the absence of aggregation) and using this concentration, the influence of the initial AntpHD-Cw3 peptide-to-lipid ratio on binding efficiency was characterized. These results, shown in Fig. 2C, demonstrate that saturation occurs around 50 μg AntpHD-Cw3/μmol liposomal lipid. Increasing the initial ratio from 60 to 80 μg AntpHD-Cw3 per μmol total lipid caused a decrease in the coupling efficiency from 80% to 65%, respectively. In comparison, liposomes made in the absence of the nickel-chelated lipid exhibited an efficiency of association of 50% at the lower (20, 40 and 60 μg) AntpHD-Cw3 to lipid starting ratio. This efficiency decreased to 40% when the initial ratio of AntpHD-Cw3 to lipid was 80 μg/μmol. It is important to note that the specificity of the binding reaction between the His-tagged AntpHD-Cw3 peptide and DOGS-NTA-Ni liposomes was confirmed by demonstrating that the binding reaction (as opposed to incorporation occurring as a consequence of hydrophobic interaction) could be inhibited in the presence of 166 mM imidazole (result not shown). In addition, it should be noted that the use of a 10-histidine tag promoted liposome–liposome cross-linking in the absence of PEG-modified lipids (Table 1). This could be explained by suggesting that the stretch of 10 histidine residues would be sufficient to bind more than one DOGS-NTA-Ni liposome. Alternatively, cross-linking could be due to the presence of multiple functional groups on the peptide such that binding due to His tag interactions could occur for one liposome while a second liposome could interact with the peptide through hydrophobic interactions. Others have demonstrated that the presence of PEG polymers inhibit surface reactions between liposomes, and can thus be used to prevent aggregation [18].

2.2. Biological activity of (His)10-AntpHD-Cw3-liposome hybrid

The results presented so far demonstrate that the AntpHD-Cw3 peptide delivery system can be easily prepared using liposomes with incorporated DOGS-NTA-Ni lipids. The procedure is rapid, simple and reproducible; however, it is important to demonstrate the protein delivery and liposome delivery attributes of the resulting formulation. Specifically, liposome-mediated delivery to APCs and subsequent

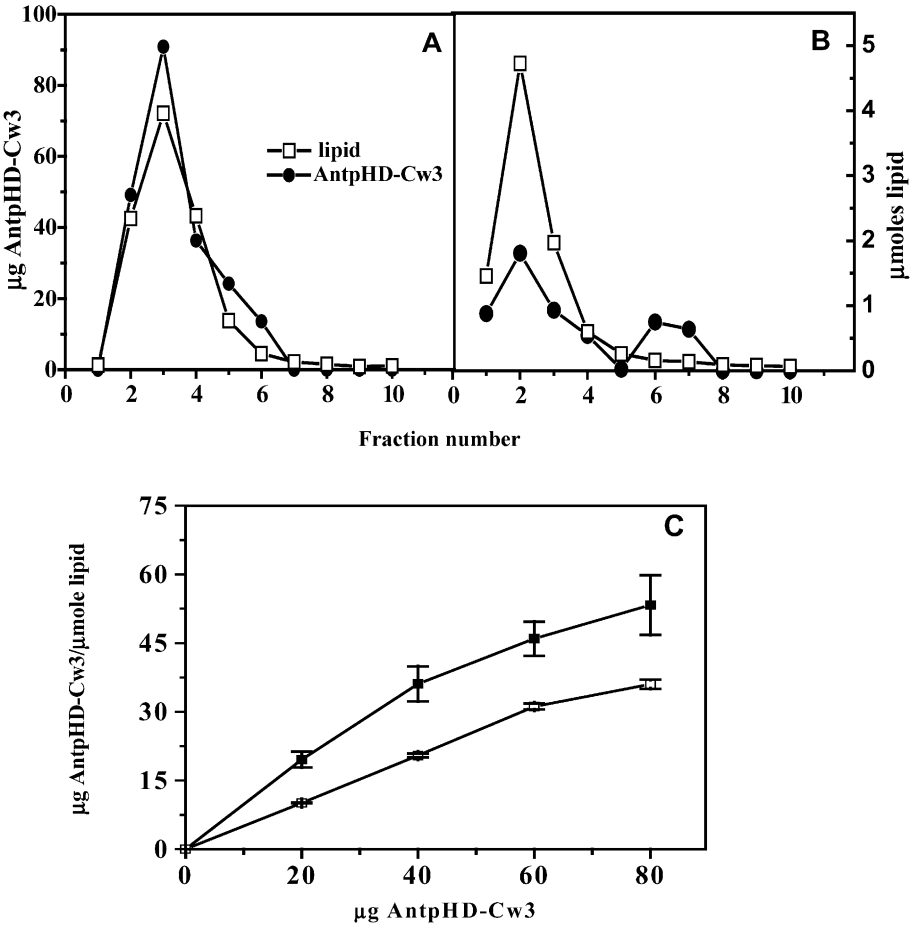

Fig. 2. Association of AntpHD-Cw3 peptide with liposomes. DOPC/DOGS-NTA-Ni (10%)/Cho/DSPE-PEG (A) and DOPC/Chol/DSPE-PEG (B) liposomes (10 µmol) were incubated with 200 µg AntpHD-Cw3. Subsequently, free peptide was separated from the associated peptide using size exclusion chromatography and the amount of peptide (●) and lipid (□) was determined for each fraction as described in Experimental protocols. The results presented are from a single representative experiment. The association of AntpHD-Cw3 as a function of initial peptide-to-liposome ratio is shown in panel C. DOPC/Chol/DSPE-PEG liposomes containing 0% (□) and 10% (■) DOGS-NTA-Ni were incubated with AntpHD-Cw3 at various peptide-to-liposome ratios for 30 min at room temperature. The amount of peptide bound was determined after the incubation period as described in Experimental protocols. Data presented represent averaged results obtained from three liposome preparations ± S.E.

AntpHD-mediated delivery of the Cw3 CTL epitope to the appropriate processing pathway should stimulate CTL. We demonstrated in a previous study that liposome-associated AntpHD-Cw3 formulations are first endocytosed by cells then trafficked through the proteasome [2]. To achieve Cw3/MHC class-I presentation to CD8+ T cells, APCs must internalize the hybrid formulation and following intracellular dissociation of the AntpHD-Cw3 peptide and delivery into the cytosol, the peptide must then enter the class I processing pathway [19]. We demonstrated this pathway for hybrid formulations prepared through methods that rely on spontaneous association of the AntpHD-Cw3 peptide with liposomes. Similarly, we assess the biological activity of formulations prepared here following immunization of mice with the peptide associated with either DOPC/DOGS-NTA-Ni/Chol/DSPE-PEG$_{2000}$ or DOPC/Chol/DSPE-PEG$_{2000}$ liposomes. Results from Elispot assays (Fig. 3), which monitor immune system stimulation by measuring the frequency of splenocytes secreting IFNγ, shows that one immunization with 50 µg AntpHD-Cw3 formulated with DOGS-NTA-Ni liposomes was able to stimulate the immune system specifically against Cw3 peptide. The frequency of splenocytes secreting IFNγ, obtained with injection of the peptide in this formulation was higher than the frequency obtained using the formulation prepared through spontaneous peptide association, however, this difference was not statistically significant. It is believed that only the antigen that is stably associated with liposomes will be able to elicit antigen-specific T cell response [20,21]. We have previously shown that AntpHD-

Table 1
Size of the formulation in the presence of various mol% of DSPE-PEG, before and after addition of AntpHD-Cw3

DSPE-PEG (%)	Size after extrusion (nm)	Size after+ AntpHD-Cw3
0	118 ± 15	>1 µm
2	120 ± 21	>1 µm
5	126 ± 22	174 ± 24 nm

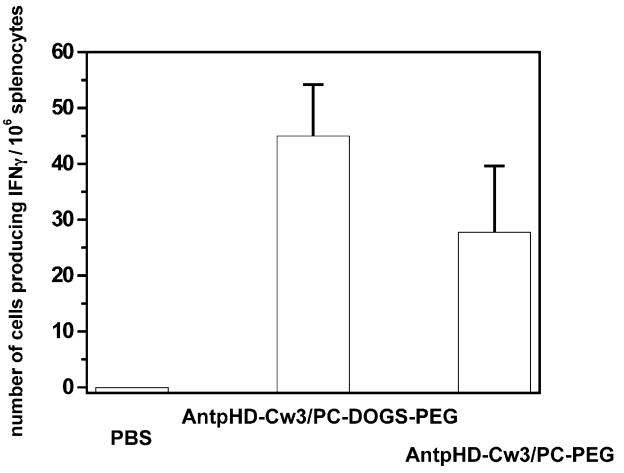

Fig. 3. Frequency of Cw3-specific T cells as determined by an IFNγ Elispot assay. BALB/c mice were immunized subcutaneously on day 0 with 50 μg AntpHD-Cw3 either in DOPC/Chol/DSPE-PEG or in DOPC/Chol/DSPE-PEG/DOGS-NTA-Ni (10%) liposomes. On day 7, spleen cells from individual mice were stimulated for 36 h with 10 μM Cw3 (170–179) and then assayed for the number of Cw3-specific cells as described in Experimental protocols. Data presented represent averaged results obtained from six mice ± S.E.

Cw3, in the absence of liposome, is unable to induce a T cell response [2]. The approach presented here allows for more efficient association of the transducing peptide than can be obtained through spontaneous binding and more efficient coupling allows a higher protein-to-lipid ratio to be achieved. Thus, a greater number of peptides are attached per liposome when using liposomes prepared with DOGS-NTA-Ni. This, in turn, will allow more peptide to be delivered with each liposome taken into the target cell population.

2.3. Coupling of a synthetic 4-histidine tagged peptide, AHNP

The results outlined above demonstrate improved coupling efficiency for a hydrophobic peptide that can spontaneously associate with liposomes, albeit at a significantly reduced efficiency, even in the absence of a coupling method. It is, however, appropriate to consider whether this procedure can be applied more generally, thus we assessed this approach using a synthetic nanomere peptide, AHNP containing a 4-histidine residue tag. AHNP is a peptidomimetic of Herceptin, an antibody with proven clinical activity against Her-2 overexpressing cancers [11]. The results, presented in Fig. 4, demonstrate rapid and efficient binding of the synthetic peptide to achieve levels of 25–30 μg of AHNP per μmol lipid. The efficiency of the coupling reaction was >60% and the dependency of coupling on the DOGS-NTA-Ni lipid was clear. In the absence of DOGS-NTA-Ni lipid, the amount of peptide bound to the DSPC/Chol liposomes at room temperature (RT) (22 °C) was insignificant, even after a 19 h incubation time. If those liposomes were prepared with 5 mol% DOGS-NTA-Ni, coupling was observed in less then 15 min and the rate of coupling was not substantially affected when the incubation temperature increased from 20 to 37 °C. Furthermore, the results summarized in Fig. 4 also demonstrate that addition of 5 mol% of DSPE-PEG_{2000} caused a slight, but measurable, change in the rate of binding (the maximum level of association was reached after 1 h as opposed to >10 min in the absence of the PEG-modified lipid), but the efficiency of incorporation did not change.

Fig. 5A demonstrates how coupling of the His-tagged AHNP peptide was affected by the initial peptide-to-liposome ratio and mol% of DOGS-NTA-Ni lipid used when preparing the liposomes. In the absence of the metal-chelating lipid, the level of peptide association was less than 4 μg of AHNP per μmol lipid, a level observed when the initial peptide-to-lipid ratio was 80 μg/μmol. For liposomes prepared with a defined amount of the DOGS-NTA-Ni lipid, AHNP association increased linearly as a function of increased AHNP levels added. Further, the level of AHNP association increased at any given peptide concentration as the mol% of DOGS-NTA-Ni incorporated in the liposomes was increased. It should be noted that these formulations could not be prepared with 10 mol% DOGS-NTA-Ni due to a DOGS-NTA-Ni-mediated aggregation reaction (data not shown). However, when using 5 mol% of the Ni-modified lipid, there was no aggregation observed in the absence of PEG-modified lipids. This was unlike the observations with His-tagged AntpHD-Cw3, and this may be due to the number of histidine residues attached to the peptide and/or the fact that AntpHD-Cw3 has hydrophobic regions. The study summarized in Fig. 5B indicates that complete inhibition of AHNP binding to the DOGS-NTA-Ni liposomes could be achieved using 60 mM imidazole. As noted earlier, 166 mM imidazole was required to inhibit the

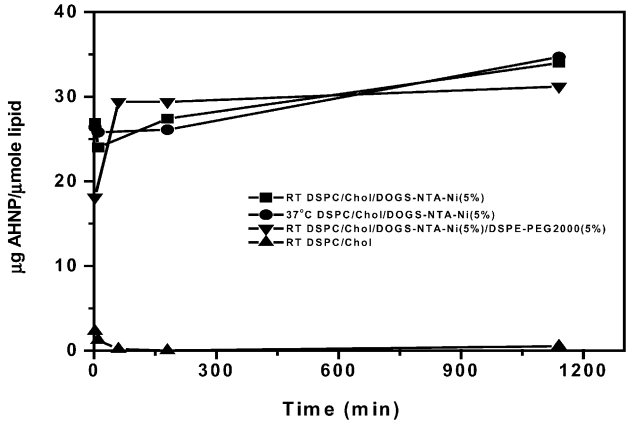

Fig. 4. Time course of His-AHNP coupling to DSPC/Chol liposomes (▲), DSPC/Chol/DOGS-NTA-Ni (5%) liposomes (■,●), and DSPC/DOGS-NTA-Ni (5%)/Chol/DSPE-PEG2000 (5%) (▼). Liposomes were incubated with His-AHNP at a peptide-to-liposome ratio of 50 μg/μmol. Unbound peptide was removed as described in Experimental protocols to determine the amount of peptide bound. Results presented are single determinations from a representative experiment.

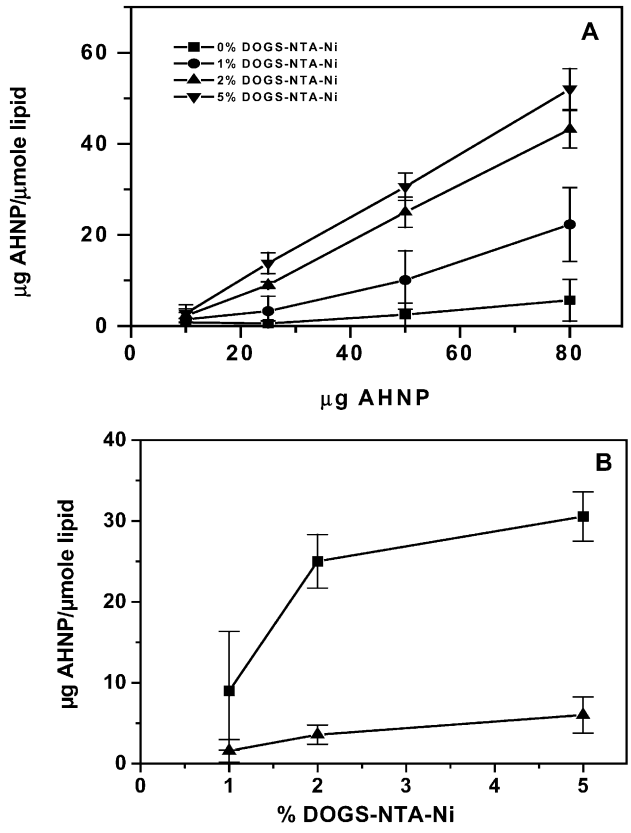

Fig. 5. Coupling of His-AHNP as a function of initial peptide-to-liposome ratio and binding specificity. (A) DSPC/Chol liposomes (1 µmol) containing 0% (■), 1% (●), 2% (▲), and 5% (▼) DOGS-NTA-Ni were incubated with various amounts of His-AHNP for 19 h at room temperature. The amount of peptide bound was determined after the incubation period as described in Experimental protocols. (B) Inhibition of AHNP binding to DSPC/Chol liposomes containing the indicated amount of DOGS-NTA-Ni lipid at 50 µg/µmol initial ratio. In the presence (▲) or in the absence (■) of 60 mM imidazole.

association of the 10 His-tagged AntpHD-Cw3 peptide to liposomes, and at an imidazole concentration of 60 mM, very weak inhibition was observed. Although not conclusive, these inhibition data suggest that binding stability may increase as the length of the His-tag used increases.

Fig. 6 illustrates that AHNP coupled to liposomes through the DOGS-NTA-Ni lipid can bind SKBR3 breast cancer cells, a cell line known to overexpress Her-2/neu as a consequence of gene amplification. AHNP-mediated binding of liposomes to cells is substantially greater than comparable formulations prepared without the peptide.

In considering the utility of this coupling approach to prepare hybrid delivery systems there are studies suggesting that nickel compounds are potentially carcinogenic [22] and, therefore, it is unlikely that the DOGS-NTA-Ni will be of value when considering the development of hybrid liposome–peptide delivery systems for human use. Having stated this, it is worth noting that acute toxicity studies in mice given three weekly i.v. or s.c. administered doses of 4 µmol DOPC/DOGS-NTA-Ni (10%)/Chol/DSPE-PEG or 3.3 µmol DSPC/DOGS-NTA-Ni (5%)/Chol i.v. resulted in no overt signs of toxicity over a 30-day time period after the third injection (results not shown). In addition, preliminary pharmacokinetic analysis of the DOGS-NTA-Ni suggested that there was little in the way of a detectable immune response to this lipid (results not shown). This does not suggest, however, that formulations with attached peptides are not immunogenic. Assuming that an alternative to DOGS-NTA-Ni will have to be identified for long-term safety reasons, it is important to understand that other transition metals such as cobalt and copper have available coordination sites and each of these coordination sites has the capacity to interact with electron-rich ligands such as histidine, tryptophane or cysteine residues [23]. While the interactions of these residues with metal ions may be relatively weak, increasing the number of amino acids used to define the affinity tag will increase binding strength. We believe that the data summarized in this report provides "proof-of-principle" support to the concept of using liposomes with DOGS-NTA-Ni to couple peptides with histidine tags. We are now exploring the use of other, perhaps safer, metal-ion-chelating lipids as alternatives to DOGS-NTA-Ni [24]. We believe that this approach, in general, is particularly suitable for preparation and characterization of recombinant and synthetic peptides engineered to have an affinity tag. This is a promising approach because of its simplicity, efficiency and the potential to control binding and dissociation of the peptides in a well-defined manner. In addition, since chemical modification of the peptide is not required after isolation, it is more likely that the functional properties of the peptide will be maintained, thus retaining the full therapeutic potential of each component of the hybrid formulations prepared using this approach.

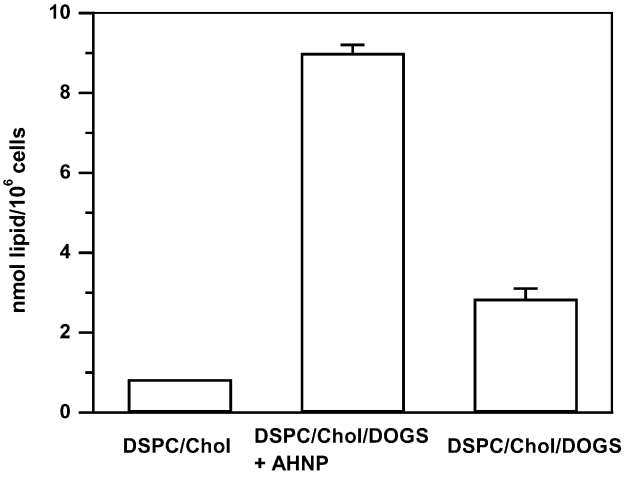

Fig. 6. Binding of AHNP associated to DOGS-NTA-Ni liposomes to SKBR3 cells. SKBR3 cells (10^6) were incubated with 1 µmol of each indicated liposome formulation at 4 °C. After 4 h incubation, cells were washed, counted and liposomal lipid quantified using scintillation counting.

3. Experimental protocols

3.1. Materials

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) lipid was purchased from Northern Lipids (Vancouver, Canada). 1,2-Distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*n*-[poly(ethylene glycol)$_{2000}$] (DSPE-PEG$_{2000}$), 1,2-dioleoyl-*sn*-glycero-3-{[*n*(5-amino-1-carboxypentyl)iminodiacetic acid]succinyl} (Nickel Salt) (DOGS-NTA-Ni) and 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) were purchased from Avanti Polar Lipids (Birmingham, AL). CHE [^{3}H] cholesterol-hexadecyl ether was purchased from Amersham (Piscataway, NJ) (1 mCi/ml). Cholesterol was obtained from Sigma (St. Louis, MO). Pico-Fluor-40 scintillation cocktail was obtained from Canberra-Packard Canada (Missasauga, ON). RPMI and DMEM media were purchased from Stem Cell Technologies (Vancouver, Canada). Elispot, multiscreen IP clear plates were obtained from Millipore (Bedford, MA). MicroBCA protein assay kit was obtained from Pierce (Rockford, IL). Centrifuge device (Microcon-30) was obtained from Millipore (Nepean, ON). Biogel 1.5 and 15 m were obtained from Bio-Rad (Hercules, CA). Peptide 170–179 HLA-Cw3 (RYLKNGKETL) and the cyclic peptide His-AHNP (HHHHFCDGFYACYMDV) were synthesized by the Biotechnology Laboratory (UBC, Vancouver, Canada).

3.2. Animals

BALB/c (H2^d) mice were obtained from the joint animal facility at the British Columbia Cancer Research Centre (Vancouver, Canada). All animal studies were completed using protocols that were approved by the Institution Animal Care committee and the methods used were consistent with the current guidelines of the Canadian Council of Animal Care.

3.3. Cell culture

SKBR3 cells were cultured in DMEM medium supplemented with 10% FBS, L-glutamine (1%), and penicillin/streptomycin (1%) at 37 °C in a humidified incubator with 5% CO_2.

3.4. AntpHD-Cw3 gene construction and synthesis

AntpHD-Cw3 fusogenic peptide initially cloned in pAH61S plasmid [25] was sub-cloned into the pET19 (Novagen, Inc., Madison, WI) between *Nde*I and *Bam*HI under the control of a T7 promoter. The peptide was expressed as a fusion peptide containing 10 histidine residues plus 13-amino acid linker attached to its amino terminus. For peptide expression, *Escherichia coli* strain BL21 (DE3) Lys was used, it contains the T7 RNA polymerase gene under control of lac promoter [26]. Peptide was purified as previously described [1], by nickel-chelate affinity resin according to the recommendations of the supplier (Qiagen, Chatsworth, CA). The eluted fractions were analyzed by SDS-PAGE on 15% gels and Coomassie blue staining. Purity was assessed at 80–90%. Before incubation with liposomes, the peptide was dialyzed against 50 mM NaH_2PO_4, 400 mM NaCl, pH 8 buffer in order to remove the imidazole, which might interfere with the peptide association with DOPC/DOG-NTA-NI/CHOL/DSPE-PEG$_{2000}$ liposomes.

3.5. Liposome preparation

Liposomes were prepared using the method described by Hope et al. [27]. Lipid mixtures used were DOPC/Chol, DOPC/Chol/DSPE-PEG$_{2000}$, DOPC/DOGS-NTA-Ni/Chol, DOPC/DOGS-NTA-Ni/Chol/DSPE-PEG$_{2000}$, DSPC/Chol, DSPC/DOGS-NTA-Ni/Chol and DSPC/DOGS-NTA-Ni/Chol/DSPE-PEG$_{2000}$ at the indicated mol% ratio. The lipids were dissolved in chloroform and trace levels of [^{3}H] cholesteryl hexadecyl ether (1–5 μCi/100 μmol total lipid) were added as a non-exchangeable and non-metabolizable liposomal lipid marker [28]. A lipid film was formed following removal of excess solvents under a stream of nitrogen gas. The lipid film was then placed under high vacuum for at least 3 h to remove all residual solvent before hydration either with HBS (100 mM HEPES; pH 7.4, 150 mM NaCl) for liposomes used for AHNP peptide or phosphate buffer (50 mM NaH_2PO_4, 400 mM NaCl, pH8) for liposomes used for AntpHD-Cw3 peptide. The resulting multilamellar vesicles were subjected to five freeze–thaw cycles [29] and then extrusion (10 times) through 100 nm pore size polycarbonate filters (Nucleopore) using an extrusion device (Northern Lipids). Liposome size was determined by quasi-elastic light scattering using Nicomp submicron particle size analyzer. All liposomal preparations exhibited average diameters of 100–120 ± 25 nm.

3.6. AntpHD-Cw3 conjugation to liposomes

Two hundred micrograms of AntpHD-Cw3 dialyzed against 50 mM NaH_2PO_4, 400 mM NaCl, pH8 buffer, was incubated with 10 μmol liposomes (v/v) for 30 min at RT under rotary shaking. The mixture was then loaded on a Biogel A 1.5 m chromatography column in order to remove the free peptide from peptide that was incorporated into the liposome. The peptide/liposome ratio was determined by quantifying liposomal lipid using [^{3}H] CHE. Radioactivity was measured by liquid scintillation counting on the Canberra-Packard Scintillation β counter (1900 TR Tri Carb), using Pico-fluor 40 scintillation cocktail. The peptide was quantified using micro-BCA protein assay kit, after dissolving the liposomes in 0.5% Triton X-100. BSA was used as the standard for this analysis.

3.7. AHNP peptide conjugation to liposomes

In the conjugation reaction, (His)4-AHNP (10–80 μg) was incubated with liposomes (1 μmol) in a total volume of 50 μl HBS (pH 7.4) at 37 °C or at room temperature for the indicated time periods as specified in each experiment. His-peptide conjugation to liposomes was quantified indirectly by measuring the amount of free peptide at the end of the conjugation reaction. Unbound peptide was separated using the Microcon-30 centrifuge device. Before centrifugation, the liposome–peptide mixture was diluted to a final volume of 250 μl in HBS. After centrifugation for 13 min at 12,000 × *g*, 20 μl of the filtrate was assayed for free peptide content using the micro-BCA assay. The amount of peptide bound to liposomes was determined by subtracting the amount of free peptide from the total amount used. This indirect quantification method of His-peptide binding to liposomes was compared with and yielded the same result as the direct method where liposome-bound peptide was directly quantified using the micro-BCA assay after separation of free peptide by size exclusion chromatography using Biogel A 15 m gel matrix (results not shown). For binding inhibition assay, 3 μl of a 1 M imidazole solution was added to the reaction mixture before the addition of liposomes.

3.8. Cell binding experiments

The SKBR3 cell line, positive for HER-2/neu, was used for liposome binding experiments. On the day of the experiment, the cells were harvested using trypsin/EDTA and then washed once using DMEM complete medium. One million cells were incubated at 4 °C for 4 h in 0.5 ml medium containing 1 mM of liposomes. To remove unbound liposomes, the cells were washed three times using 3.5 ml of PBS and centrifuged for 5 min at 1500 rpm for each wash. The cells recovered after washing were then counted using 5 ml of Pico-Fluor 40 scintillation cocktail and using a Beckman LS 3801 scintillation counter.

3.9. Immunization

Groups of BALB/c mice (six per group), 6–8 weeks of age, were immunized s.c. once. On day 7, spleens were harvested and the immune response was monitored using an Elispot as described bellow.

3.10. Elispot

We have used the same the Elispot assay protocol described by Chikh et al. [2]. Briefly, Elispot plates (Multiscreen-IP Clear plates, Millipore) were coated overnight at 4 °C with capture anti-INFγ antibody (2 μg/ml) (clone R4-6A2) (Pharmingen). The plates were then blocked with 1% BSA in PBS for 2 h at RT. After three washes, responder cells in RPMI medium supplemented with 10% ConA supernatant containing TCGF (T cell growth factors), 10% FCS, 1% glutamine, 1% penicillin/streptomycin and 5×10^{-5} M β-2-ME (mercaptoethanol) were added to the wells along with 5×10^5 irradiated syngeneic feeder cells. Cells were incubated for 36 h in the presence or absence of 10 μM of Cw3 (170–179) peptide. After culture, the plates were washed and biotinylated anti-INFγ detection antibody (clone XMG1.2) (Pharmingen) was added (1 μg/ml) and the plates were then incubated for 1 h at RT. Spots developed following addition of freshly prepared HRP diluted 1:2000 in PBS/Tween containing 1% BSA, followed by repeated (5 ×) washes with PBS/Tween and addition of 200 μl of HRP substrate (Opti-4CN substrate kit, Bio-Rad). The frequency of peptide-specific T cells was calculated based on the percentage of cells present in the responding population.

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