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Authors

Hendricks, Gabriel

Velazquez, Lourdes

Pham, Serena

et al.

Publication Date

2015-04-01

DOI

10.1016/j.antiviral.2015.01.008

Peer reviewed

HHS Public Access

Author manuscript

Antiviral Res. Author manuscript; available in PMC 2016 August 17.

Published in final edited form as:

Antiviral Res. 2015 April ; 116: 34–44. doi:10.1016/j.antiviral.2015.01.008.

Heparin octasaccharide decoy liposomes inhibit replication of multiple viruses

Gabriel L. Hendricks[a,2], **Lourdes Velazquez**[b], **Serena Pham**[a], **Natasha Qaisar**[a], **James C. Delaney**[c], **Karthik Viswanathan**[c], **Leila Albers**[d], **James C. Comolli**[d], **Zachary Shriver**[c], **David M. Knipe**[e], **Evelyn A. Kurt-Jones**[a], **Deborah K. Fygenson**[b], **Jose M. Trevejo**[d], **Jennifer P. Wang**[#a,*], and **Robert W. Finberg**[#a]

[a] Department of Medicine, University of Massachusetts Medical School, 364 Plantation Street, Worcester, MA 01605, United States

[b] Department of Physics, Broida Hall, University of California, Santa Barbara, CA 93106, United States

[c] Department of Biological Engineering, Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, 500 Main Street, Cambridge, MA 02142, United States

[d] Charles Stark Draper Laboratory, Department of Bioengineering, 555 Technology Square, Cambridge, MA 02139, United States

[e] Department of Microbiology and Immunobiology, Harvard Medical School, Boston, MA 02115, United States

[#] These authors contributed equally to this work.

Abstract

Heparan sulfate (HS) is a ubiquitous glycosaminoglycan that serves as a cellular attachment site for a number of significant human pathogens, including respiratory syncytial virus (RSV), human parainfluenza virus 3 (hPIV3), and herpes simplex virus (HSV). Decoy receptors can target pathogens by binding to the receptor pocket on viral attachment proteins, acting as ‘molecular sinks’ and preventing the pathogen from binding to susceptible host cells. Decoy receptors functionalized with HS could bind to pathogens and prevent infection, so we generated decoy liposomes displaying HS-octasaccharide (HS-octa). These decoy liposomes significantly inhibited RSV, hPIV3, and HSV infectivity *in vitro* to a greater degree than the original HS-octa building block. The degree of inhibition correlated with the density of HS-octa displayed on the liposome surface. Decoy liposomes with HS-octa inhibited infection of viruses to a greater extent than either full-length heparin or HS-octa alone. Decoy liposomes were effective when added prior to infection or following the initial infection of cells *in vitro*. By targeting the well-conserved receptor-binding sites of HS-binding viruses, decoy liposomes functionalized with HS-octa are a promising therapeutic antiviral agent and illustrate the utility of the liposome delivery platform.

[*]Corresponding author. Tel.: +1 508 856 8414. Jennifer.wang@umassmed.edu (J.P. Wang).
[2]Current address: Rhode Island Hospital, 593 Eddy Street, Providence, RI 02903, United States.

Keywords

Heparin; Parainfluenza virus; Herpes simplex virus; Respiratory syncytial virus; Decoy; Liposome

1. Introduction

Numerous pathogens, including viruses, bacteria, and protozoans, use glycosaminoglycans such as heparan sulfate (HS), which are long, unbranched, disaccharide-repeating polysaccharides found throughout the body, as either an attachment molecule or a co-receptor for cell entry (Bernfield et al., 1999; Zhu et al., 2011). More than 25 viruses utilize HS for attachment or entry, including the paramyxoviruses respiratory syncytial virus (RSV) and human parainfluenza virus 3 (hPIV3), as well as the alpha herpesvirus herpes simplex virus 1 (HSV-1). Development of improved therapeutic agents for such viruses is warranted. HS is a promising candidate for use as a decoy receptor. Decoy receptors have been shown to attenuate infections by diverting the targeted pathogen away from susceptible tissues in several infection models (Asher et al., 2005; Dennehy et al., 2007; Hendricks et al., 2013; Zibert et al., 1992).

HS has a high negative charge density from extensive sulfation of its disaccharide repeat, and many viruses have evolved positive or polar amino acid HS-binding domains (HBDs) that bind tightly to HS. Viruses rely on many low affinity, multivalent interactions to bind tightly to a host target cell (Kiessling and Pohl, 1996). Viruses that bind to HS often have HBDs on more than one surface glycoprotein and multiple HBDs may be present on each protein. HBDs can be linear amino acid domains, such as glycoprotein G of RSV (Feldman et al., 1999), or non-linear positive amino acids on exposed loops of a surface protein, such as the fusion protein of human metapneumovirus (HMPV) (Chang et al., 2012). HS-binding viruses require HS on the cell surface for initial attachment, as *in vitro* studies demonstrate that they show reduced binding to cells lacking HS. Removal of HS reduced infection rates between 75% and 98% for RSV (Feldman et al., 2000; Krusat and Streckert, 1997), ~90% for HMPV (Adamson et al., 2012; Thammawat et al., 2008), and 95% for HSV (WuDunn and Spear, 1989), even though the protein receptor for each virus was present.

Decoy receptors act as ‘molecular sinks’, binding to viruses to reduce circulating titers over the course of infection. Decoys may make individual low affinity interactions with a particular pathogen, but multimerization generates many more interactions, increasing the overall avidity of the virus–decoy interaction. This principle is illustrated with sialic acid decoys for influenza virus (Gambaryan et al., 2002; Hendricks et al., 2013; Mammen et al., 1995). HS-binding viruses can be inhibited with soluble heparin, a natural polymer that acts as a decoy. The 50% inhibitory concentration (IC_{50}) of full-length heparin for RSV is typically in the sub-micromolar range (~0.05 μM) (Donalisio et al., 2012; Krusat and Streckert, 1997). Low molecular weight (LMW) heparin, which contains shorter disaccharide chains, can also inhibit RSV infectivity, but is about 10-fold less effective than full-length heparin (Feldman et al., 2000). When used against HMPV, another paramyxovirus, the IC_{50} for soluble heparin is 1 μM (Thammawat et al., 2008). hPIV3 binds to both α2–3 linked sialic acid (Amonsen et al., 2007) and HS. Heparin inhibition of hPIV3

is less dramatic as the IC_{50} is only 66 μM (Bose and Banerjee, 2002). For both HSV-1 and HSV-2, full-length heparin has an IC_{50} of <1 μM HS (WuDunn and Spear, 1989) and is more inhibitory compared to either LMW heparin or very short trito penta-disaccharide heparin repeats (Nyberg et al., 2004). Thus, the length of each decoy heparin chain correlates with its inhibitory capacity, underscoring the role of multivalency. Unfortunately, the anticoagulant activities of full-length and LMW heparin make them unsuitable as antiviral agents.

Liposomes are a promising method of delivering decoy receptors. We and others previously demonstrated that incorporation of a monovalent decoy receptor for sialic acid into a fluid liposome increased its affinity to influenza virus relative to the original monovalent building block (Guo et al., 2002; Hendricks et al., 2013; Kingerywood et al., 1992). Lateral movement through the liposome membrane allows for proper decoy presentation to different saccharide-binding proteins, which have unique spatial arrangements for particular viruses. Here, we further extend the decoy liposome platform beyond sialic acid by generating liposomes containing heparin octasaccharide (HS-octa). HS-octa chains consist of four disaccharide repeats that are derived from digested commercial heparin. We demonstrate that decoy liposomes containing HS-octa inhibit infectivity of RSV, HSV-1 and hPIV3. Thus, decoy liposomes can be generated to target diverse viruses that share a common host ligand and serve as an adaptable platform capable of targeting any pathogen that binds to a defined moiety.

2. Materials and methods

2.1. Production of octasaccharides

Full-length heparin (Celsus Laboratories, Cincinnati, OH) was subjected to partial digestion using enzymatic means. Enzymatic digestion was completed with recombinant heparinase I from *Flavobacterium heparinum* overnight at 25–30 °C in 50 mM ammonium acetate buffer, pH 5.0, with 1 mM calcium.

2.2. Chromatography

Digested polysaccharides were size-separated using fast protein liquid chromatography (Fig. 1A). Prior to size separation, the sample was lyophilized and then dissolved at 10 mg/mL. A 5 mL sample was injected onto a Superdex 30 column with a running buffer of 5 mM Na_2HPO_4 and 150 mM NaCl at a pH of 7.2. The separated chains were detected at 232 nm with a UV detector. Fractions of octasaccharide were collected, buffer-exchanged into 10% ethanol, and lyophilized to a dry powder. The purity of each size-separated fraction was verified using analytical gel permeation chromatography. To achieve higher resolution, a second small-scale purification step was performed using a semi-preparative scale CarboPac PA-200 high performance liquid chromatography (HPLC) column (9 mm dia. × 250 mm length). Partially purified octasaccharide (2.5 mg per run) was loaded on the HPLC and the desired separation was achieved with a 650–1250 mM NaCl gradient run at 2.5 mL/min.

2.3. Glycolipid synthesis

Glycolipid synthesis is schematically represented in Fig. 1B. 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) (Avanti Polar Lipids, Alabaster, AL) was attached to a Fmoc-aminooxyacetic acid (Chem-Impex International, Wood Dale, IL) using carbodiimide coupling and purified by 60 Å silica gel column chromatography. Piperidine (Sigma–Aldrich, St. Louis, MO) was used to remove the Fmoc from the lipid-linker conjugate and the purity was determined by MALDI-MS. Heparan sulfate octasaccharide was conjugated at room temperature with 3 mol% acetic acid in a mixture of water and tetrahydrofuran. The final HS-octa glycolipid product was purified by HPLC to obtain heparan sulfate-containing glycolipids. HS-octa was quantified by UV_{232} absorbance and characterized by MALDI-mass spectrometry (Fig. 1C). Strong anion exchange HPLC along with proton NMR (Fig. 1D) confirmed the presence of heparan sulfate octasaccharide in the glycolipid.

2.4. Anti-coagulant activity

Purified samples were tested for anti-factor Xa activity using the Chromogenix Coatest Heparin kit (Diapharma, West Chester, OH) on a COAG-A-MATE MTXII instrument (Organon Teknika Corporation, Durham, NC) according to manufacturers' protocols.

2.5. Liposome preparation

Gas-tight syringes (Hamilton Co., Reno, NV) and vials (National Scientific, Rockwood, TN) were thoroughly cleaned, rinsed 10× with 100% ethanol and then 10× with chloroform. Vials were soaked in 300 mM HCl for 1.5 h, and then rinsed thoroughly with water, 3× with ethanol and 3× with chloroform. Residual solvent was evaporated under a filtered stream of dry nitrogen gas.

To compensate for the net negative charge on HS-octa-containing decoy liposomes, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), a zwitterionic, non-reactive phospholipid, was incorporated in some liposome formulations so that all liposomes contained 30% negatively charged lipids. Control liposomes contained 30 mol% 1,2-dioleoyl-*sn*-glycero-3-phospho-*rac*-(1-glycerol) sodium salt (DOPG), a negatively charged, 18:1 fatty acyl chain lipid, in place of the negatively charged HS-octa-DOPE (see Table 1).

Lipids were mixed and prepared as previously described (Hendricks et al., 2013). Lipids were extruded by 21 passes through a polycarbonate membrane using a LiposoFast-Basic Extruder, equipped with a 200 nm pore filter (Avestin, Ottawa, ON, Canada). The extruder was thoroughly cleaned and primed with buffer before use. After the final pass, samples were collected in a clean vial, sealed with a Teflon-lined cap and stored at 4 °C until use.

Lipid concentration post-extrusion relative to pre-extrusion was determined by fluorimetry. Concentrations were estimated based on mass lipid deposited and volume hydrated. Typical recoveries were ~80%. Samples were hydrated to an estimated concentration of 1 or 5 mM total lipid.

2.6. Liposome characterization

Diameter and polydispersity of liposomes were determined by dynamic light scattering (Zetasizer Nano, Malvern Instruments, Worcestershire, UK) with specifications for a lipid refractive index of 1.480 and a dispersant (150 mM PBS) refractive index of 1.332. Measurements were obtained using 40 μL disposable cuvettes at room temperature (20 °C) and a backscattering angle of 173°. Representative values are listed in Table 1. The low polydispersity index (PDI) indicates that the decoy liposomes are a relatively uniform population of particles. Decoy liposomes were stored at 4 °C. The molarity of HS-octa for liposomes was calculated by multiplying the input mol% HS-octa-DOPE lipid by the total lipid concentration post-extrusion.

2.7. Viral strains

Human RSV strain A2 was grown in Vero cells obtained from the American Type Culture Collection (ATCC, Manassas, VA) as previously described (Murawski et al., 2009). hPIV3 was grown in LLC-MK2 cells (ATCC) and cultured in Eagle's minimum essential medium (EMEM) containing 10% fetal bovine serum (FBS), 1% penicillin–streptomycin (Pen/Strep), 1% non-essential amino acids, and 1% sodium pyruvate. hPIV3 was propagated in hPIV3 growth medium [DMEM-F-12 medium mixed 1:1 with 1× ITS universal culture supplement (BD Biosciences, San Jose, CA)] and 1 μg/mL TPCK trypsin. After 30 min at 37 °C, additional growth medium was added and cells were incubated for 4 to 6 days at 37 °C, 10% CO_2 until a high cytopathic effect was observed. Supernatants were collected and clarified by centrifugation at 300×*g* for 15 min then stored at −80 °C prior to use in infectious studies. For hemagglutination inhibition studies hPIV3 was concentrated by polyethylene glycol (PEG)-based precipitation then clarified supernatants were mixed 4:1 with 50% (w/v) PEG-6000 and stored at 4 °C overnight prior to centrifugation at 1000×*g* for 45 min. The resulting pellet was resuspended in 1/10 of the starting volume and stored at −80 °C. HSV-1 strain KOS was grown and titrated in Vero cells as described previously (Knipe and Spang, 1982). All viruses were stored at −80 °C prior to use.

2.8. Hemagglutination inhibition assay

Red blood cells (RBCs) were isolated from healthy human volunteers, as described previously (Hendricks et al., 2013). Briefly, whole blood was washed in sterile PBS, diluted 1:30 in sterile PBS, and stored at 4 °C prior to use. All procedures involving human subjects were approved by the University of Massachusetts Medical School's Committee for the Protection of Human Subjects in Research and in accordance with the Declaration of Helsinki.

The hemagglutination (HA) titer for each aliquot was determined on RBCs prior to each inhibition study per standard protocol (Choi et al., 1996). For hemagglutination inhibition (HAI) assays, liposome samples were serially diluted two-fold in PBS. Four HA units of hPIV3 in 25 μL of PBS were added to all dilutions. Samples were incubated for 30 min at room temperature. 50 μL of diluted RBCs were added to the wells and incubated for an additional hour at 4 °C to allow for agglutination. The HAI titer is the reciprocal of the greatest dilution of liposomes that results in non-agglutinated RBCs.

2.9. Viral infectivity assays

For RSV infectivity assays, Vero cells were seeded into 24-well plates and incubated at 37 °C, 10% CO_2 for 24 h to form monolayers. RSV, diluted to 300 plaque forming units (PFU)/mL, was mixed 1:1 (v/v) with liposome samples serially diluted to the desired test concentrations in DMEM. The virus/liposome mixtures were pre-incubated together at 37 °C for 30 min. Cells were washed with PBS, then the virus–liposome mixtures were added to cells in duplicate wells. Incubation continued at 37 °C, 10% CO_2 for 1 h to allow for infection. The liposome–virus mixture was removed and then cells were washed with PBS and incubated with DMEM containing 10% FBS at 37 °C, 10% CO_2 for 72 h. Cells were fixed and stained with anti-glycoprotein F and anti-glycoprotein G antibodies (MAB858-2 and MAB8262F, Millipore, Billerica, MA). Plaques were visualized with anti-mouse horseradish peroxidase-conjugated secondary antibody (BD Biosciences, San Jose, CA) and developed with peroxidase substrate kit (Vector Laboratories, Burlingame, CA). Viral plaques in the cell monolayer were quantified.

HSV infectivity assays were performed on Vero cells (seeded into 96-well plates) in a similar fashion as for RSV, except after 1 h of incubation with virus–liposome mixtures, Vero cells were washed with PBS and incubated in DMEM containing 100 μg/mL pooled human IgG (Innovative Research, Novi, MI) for 48 h at 37 °C, 10% CO_2. Cells were fixed and stained with 20% ethanol with 0.4% crystal violet in PBS and plaques were counted.

For hPIV3 infectivity assays, LLC-MK2 cells were seeded into 6-well plates and incubated overnight at 37 °C, 5% CO_2 to form monolayers. hPIV3, diluted to 50 PFU/mL, was mixed 1:1 (v/v) with liposome samples serially diluted to the desired concentrations in sterile EMEM. These virus-liposome samples were co-incubated together at 37 °C for 30 min. Cells were washed with PBS and virus–liposome mixtures were added to cells in duplicate wells and incubated for 1 h at room temperature to allow for infection. LLC-MK2 cells were washed with PBS and incubated with 0.8% agarose and 3 μg/mL acetylated trypsin in EMEM at 37 °C, 5% CO_2 for 8 days. Cells were fixed and stained with 0.5% crystal violet in 4% paraformaldehyde.

2.10. Viral replication assays

For RSV, Vero cells were seeded into 24-well plates and grown to confluency overnight at 37 °C, 10% CO_2. RSV, diluted to a MOI of 1, was mixed 1:1 (v/v) with liposome samples serially diluted to the desired test concentrations in DMEM. The virus/liposome mixtures were pre-incubated together at 37 °C for 30 min. Vero cell monolayers were washed with PBS and cells were infected with the liposome–virus mixture for 1 h at 37 °C, 10% CO_2. [NB: For a subset of experiments, cells were first infected with RSV (MOI 1). Without any wash or removal of virus, decoy liposomes (1:1, v/v) were added to duplicate wells and the plate was swirled to mix. Infection was continued at 37 °C for 1 h.] Following incubation, the liposome–virus mixture was removed and cells were washed with PBS. Viral growth medium containing fresh liposomes was added. After incubation for 48 h at 37 °C, 10% CO_2, supernatants were collected and virus growth was quantified by plaque assay.

For HSV replication assays, a similar protocol was followed. Vero cells were plated in 96-well plates and grown to confluency overnight at 37 °C, 10% CO_2. HSV (MO1 0.1) and liposomes were first pre-incubated together for 30 min at 37 °C, then added to freshly washed cells and incubated for 1 h at 37 °C, 10% CO_2. [NB: For a subset of experiments, cells were infected with HSV-1 (MOI 0.1), diluted liposomes (1:1, v/v) were immediately layered on top, and cells were incubated for 1 h at 37 °C, 10% CO_2.] After the 1 h infection period, cell monolayers were washed with PBS, and DMEM/10% FBS containing the fresh liposomes was added back to appropriate wells. Supernatants were sampled at 48 h and stored at −80 °C for subsequent assessment of virus growth by plaque assay.

2.11. Statistics

Statistical analysis was performed using the unpaired, two-tailed Student's *t*-test. Values of $P < 0.05$ were considered significant. Error bars are ± standard error of the mean (S.E.M.). Nonlinear curve fitting and statistics were calculated using Prism Version 6.0 (GraphPad Software).

3. Results

3.1. Synthesis of heparin octasaccharide and heparin-octasaccharide liposomes

The heparin saccharide derivative HS-octa contains four disaccharide repeats. Unlike heparin, these short saccharides do not have any anticoagulant property thus making them a potential antiviral therapeutic agent. HS-octa retains the capacity to bind to thrombin (Sidhu et al., 2013) but does not bind to anti-thrombin (Olson et al., 1991). Without thrombin and anti-thrombin bridging, HS-octa does not aid in anti-thrombin transformation, and therefore does not result in prolongation of partial thromboplastin time, inhibition of factor Xa, or inhibition of factor IIa in the anti-coagulation cascade (Hasan et al., 2005). We found that heparin's anti-factor Xa activity is ~150 IU/mg, while HS-octa's anti-factor Xa activity is 0–10 IU/mg, depending on the preparation. HS-octa was conjugated to DOPE (HS-octa-DOPE), which contains a reductive primary amine, to facilitate the addition of molecules with distinct linker chemistries and orientations to the octasaccharide (Fig. 1 and Materials and Methods). HS-octa-DOPE was mixed with DOPC, a zwitterionic, non-reactive phospholipid, to generate liposomes. DOPE and DOPC each have two 18:1 fatty acyl chains that give the liposomes a low gel–liquid phase transition temperature. The liposomes form relatively disordered lipid bilayers, allowing for HS-octa to migrate through the membrane (Jost and Griffith, 1982). We synthesized decoy liposomes with increasing amounts of HS-octa-DOPE, from 3 to 30 mol% as well as control liposomes that contained DOPG (Table 1).

3.2. Decoy liposomes containing HS-octa inhibit RSV infectivity

To assess the impact of decoy liposomes on RSV infectivity, RSV was initially co-incubated with either HS-octa decoy liposomes or control liposomes lacking HS-octa (see Fig. 2 for schematic). The combined virus/liposome mixture was added to Vero cell monolayers for 1 h. Following infection, the virus/liposome mixture was removed and RSV infectivity was assessed by plaque assay. We tested a series of liposome formulations containing various densities of HS-octa, ranging from 0 to 30 mol% HS-octa. The HS-octa molarity in solution

was calculated by multiplying the mol% input of HS-octa-DOPE lipids by the total lipid concentration. The relative infectivity was calculated as the ratio of PFU/mL for a sample versus its control and expressed as percentage infectivity.

All HS-octa-containing decoy liposomes inhibited RSV infectivity to some degree, and a strong dose response was observed (Fig. 3). The degree of inhibition correlated to the density of HS-octa. Decoy liposomes with 3 mol% HS-octa were least effective at inhibiting RSV infectivity (Fig. 3, diamonds) while decoy liposomes with 7.5 mol% HS-octa (Fig. 3, triangles) inhibited RSV significantly better than free HS-octa on a molar basis. The 10 mol % HS-octa liposomes blocked RSV infectivity to a significantly greater degree than either 3 or 7.5 mol% HS-octa liposomes (Fig. 3, circles). Maximal (>98%) inhibition of RSV infectivity was achieved with 30 mol% HS-octa liposomes (Fig. 3, Xs). These data demonstrated that incorporation of HS-octa into liposomes increases its efficacy for RSV inhibition, presumably by allowing glycolipid migration through the bilayer and increasing the avidity by multimerization of binding motifs, thereby allowing for multivalent virus–decoy interactions. Control liposomes, which contain only DOPC and DOPG lipids, were used at equimolar lipid concentrations present in the highest concentration of decoy liposomes here and in all subsequent experiments, and had no effect on RSV infectivity, as similar titers were recovered whether RSV was mixed with control liposomes or assay diluent (data not shown). Free HS-octa was only effective at blocking RSV infectivity at the highest concentration tested (Fig. 3, closed gray squares).

3.3. Decoy liposomes containing HS-octa inhibit RSV replication

To further assess the capacity of decoy liposomes to inhibit RSV during multiple rounds of infection, host cell monolayers were treated with a mixture containing RSV that had been pre-complexed with free HS-octa or liposomes (decoy liposomes with the indicated concentrations of HS-octa or control liposomes) for 30 min. After 1 h, the virus–liposome mixture was removed and replaced with fresh decoys alone. Viral replication and spread of the virus in the culture in the presence or absence of liposomes was assessed by plaque assay. The 10 mol% HS-octa (Fig. 4A, circles) and 30 mol% HS-octa (Fig. 4A, Xs) decoy liposomes at 1 μM HS-octa each inhibited viral titers compared to control liposomes (Fig. 4A, open square). Higher concentrations of 30 mol% HS-octa decoy liposomes increased inhibition of RSV replication. Free HS-octa (Fig. 4A, gray squares) inhibited RSV replication to a lesser extent than the HS-octa decoy liposomes. Notably, more than 10-fold higher concentrations of free HS-octa were required to inhibit RSV to the same degree as 30 mol% HS-octa decoy liposomes.

Inhibition was also observed when decoy liposomes were added to cells after virus infection was established. Vero cells were first infected with RSV, then liposomes were added and infection was allowed for one hour. Cells were washed and fresh liposomes were added back for the duration of the two-day culture. Decoy liposomes with 30 mol% HS-octa (Fig. 4B, Xs) significantly reduced RSV titers compared to control liposomes. Under these conditions, free HS-octa was not inhibitory (Fig. 4B, gray squares), again indicating that HS-octa liposomes are more effective than free HS-octa.

3.4. Decoy liposomes containing HS-octa inhibit infectivity of HSV-1

Several herpesviruses, including HSV-1, have been shown to bind to heparin (WuDunn and Spear, 1989), so we tested if HS-octa liposomes could inhibit HSV-1 infectivity. HS-octa decoy liposomes or control liposomes were co-incubated with HSV-1 and then used to infect Vero cells. After one hour, the liposome–virus mixture was removed and media containing pooled human IgG was added to neutralize any free virus. Viral infection was quantified by plaque assay. As with RSV, HS-octa decoy liposomes worked in a dose-dependent manner against HSV-1. On a molar basis, the HS-octa decoy liposomes inhibited HSV-1 to a greater degree than free HS-octa. Decoy liposomes containing 10 mol% HS-octa (Fig. 5, circles) at concentrations as low as 0.01 μM HS-octa inhibited HSV-1 infectivity. Maximal inhibition was observed with 30 mol% HS-octa decoy liposomes (Fig. 5, Xs), and 10 μM HS-octa was inhibitory to a similar degree as 0.01 μM HS-octa of 10 mol% decoy liposomes. Control liposomes did not affect HSV-1 infectivity, as the PFU number was comparable to that with assay diluent (data not shown). Free HS-octa was only significantly inhibitory at very high concentrations (Fig. 5, gray squares). Altogether, these data demonstrate that for both HSV-1 and RSV, HS-octa decoy liposomes are significantly more effective inhibitors than free HS-octa.

3.5. HS-octa decoy liposomes inhibit HSV-1 replication

To further test the inhibitory capacity of decoy liposomes, we examined their effect on HSV-1 infection during multiple rounds of replication. Liposomes were pre-complexed with HSV-1 and added to Vero cells. After 1 h, the virus/liposome mixtures were removed, cells were washed, media containing fresh liposomes were added, and cells were incubated for another 48 h. Decoy liposomes with 30 mol% HS-octa were added to HSV-1-infected Vero cells at various concentrations. Decoy liposomes with 30 mol% HS-octa were highly effective under these conditions (Fig. 6), as HSV-1 titers were significantly reduced in comparison to control liposomes (Fig. 6A, Xs). Concentrations above 0.01 μM 30 mol% HS-octa decoy liposomes resulted in undetectable HSV-1 titers (>99% inhibition). Free HS-octa (Fig. 6A, gray squares) inhibited replication by less than 10-fold at the highest concentration tested.

Inhibition of HSV-1 replication was also observed when virus and decoy liposomes were added separately to Vero cells. Vero cells were infected with HSV-1 and liposomes were immediately added. After 1 h, cells were washed and liposomes were added back at the original concentration for 48 h. At the highest concentrations tested (1 μM HS-octa), decoy liposomes with 30 mol% HS-octa reduced HSV-1 titers to below the limit of detection (Fig. 6B, Xs). At lower concentrations, decoy liposomes continued to inhibit HSV-1 growth compared to control liposomes. The inhibition seen was dose-dependent. In contrast, free HS-octa (Fig. 6B, gray squares) did not inhibit HSV-1 as well as HS-octa decoy liposomes. Approximately 100-fold higher concentrations of free HS-octa were required to inhibit HSV replication compared to liposomal HS-octa. These data suggest that HS-octa decoy liposomes not only inhibit the initial infection of the HS-binding viruses, but may also prevent cell-to-cell spread, and are much more effective than free HS-octa.

3.6. HS-octa decoy liposomes competitively inhibit human parainfluenza virus 3 hemagglutination and infectivity

Next, we tested the effectiveness of HS-octa decoy liposomes against hPIV3. Viral hemagglutination results when viruses attach to molecules present on the surface of RBCs, causing the RBCs to form a lattice. hPIV3 exhibits hemagglutination (Clements et al., 1991), but HS-octa decoy liposomes inhibit hemagglutination when present in sufficiently high concentrations (Table 2). The degree of inhibition can be expressed as either the HAI titer, which is the reciprocal of the last dilution of decoy liposomes to inhibit hemagglutination, or as the molarity of HS-octa that results in HAI. Decoy liposomes inhibited hPIV3 hemagglutination, while control liposomes with equal lipid concentrations but lacking HS-octa did not inhibit hemagglutination (Table 2). Decoy liposomes with 30 mol% HS-octa were more effective at inhibiting hPIV3 hemagglutination than free HS-octa.

hPIV3 was then co-incubated with HS-octa-containing decoy liposomes, control liposomes, or free HS-octa prior to infection of LLC-MK2 cell monolayers. 30 mol% HS-octa decoy liposomes inhibited hPIV3 infectivity in a concentration-dependent manner, while control liposomes had no effect on infectivity (Fig. 7). At 10 μM HS-octa, decoy liposomes significantly inhibited infectivity (Fig. 7, Xs). In contrast, greater than 100 μM free HS-octa was required for inhibition of hPIV3. Altogether, these data demonstrate that the decoy liposomes can be used to inhibit multiple viruses that bind to HS.

3.7. Decoy liposomes inhibit HS-binding viruses more effectively than full-length heparin

Full-length heparin acts as potent inhibitor of HS-binding viruses by making multivalent interactions with viruses. We compared the inhibition of HS-binding viruses incubated with either commercial heparin, a heterogeneous mixture of oligosaccharide chains averaging ~120 saccharide repeats, or decoy liposomes containing HS-octa. For RSV, HSV-1, and hPIV3, the IC_{50} values for 30 mol% HS-octa decoy liposomes were lower than the IC_{50} values for full-length heparin (Table 3). Decoy liposomes were more effective than full-length heparin at inhibiting hPIV3 hemagglutination. HS-octa decoy liposomes had an HAI value approximately 10-fold lower than full-length heparin (Table 2), even though full-length heparin contains many more saccharide repeats than HS-octa complexed into decoy liposomes. Incorporation of HS-octa into liposomes likely increases the efficacy of the HS repeats, allowing liposomes with short octasaccharide chains to create multivalent interactions with viruses and inhibit infection to a greater degree than either free HS-octa or full-length heparin.

4. Discussion

Our results indicate that decoy receptors complexed into liposomes can sequester multiple viruses away from susceptible cells, reducing infection and potentially altering the disease course. These results support the potential use of decoy receptors as therapeutic antiviral agents. The use of a pathogen's natural receptor ligand (or a close homolog) can result in attenuation of infection and improved disease outcome. For example, we previously demonstrated that RBCs transgenically expressing the Coxsackie B virus receptor bind to and sequester Coxsackie B virus away from susceptible tissues, improving survival in mice

(Asher et al., 2005). We also previously demonstrated that incorporation of a sialic acid-based decoy receptor into liposomes resulted in inhibition of influenza A virus and colocalization of decoy–virus complexes (Hendricks et al., 2013). Previous studies have shown that monomeric decoys, or molecules that can have only one binding motif, inhibit poorly, with IC_{50} values in the millimolar range (Glick et al., 1991). However, polymerized multimeric decoy receptors have a much higher apparent affinity for viral attachment proteins and potently inhibit infectivity (Lees et al., 1994; Mammen et al., 1995). This effect of polymerization most likely results from synergistic, multiple low-affinity receptor–ligand interactions between a decoy molecule and a virus particle. Furthermore, incorporation of monomeric decoy molecules into liposomes results in a further increase of efficacy compared to the same concentration of soluble decoys (Guo et al., 2002; Hendricks et al., 2013; Kingerywood et al., 1992).

HS-octa decoy liposomes are significantly more effective than free HS-octa at inhibiting three distinct HS-binding viruses. HS-octa contains fewer binding motifs per molecule than full-length heparin, reducing the potential valency or number of possible virus–decoy interactions per molecule. This reduction in binding motifs correlates to a reduction of effectiveness of free HS-octa compared to full-length heparin. However, the antiviral properties of HS-octa decoy liposomes suggest that the incorporated HS-octa molecules act synergistically as one polymer-like particle. The effect is more than additive, as the inhibition is dependent not only on the concentration of total HS-octa in the solution, but also on the density for each individual liposome. Liposomes allow for lateral diffusion of decoy molecules across the surface of the liposome (Jost and Griffith, 1982) so that the optimal binding conformation may occur between virus and decoy. Escalating the concentration of the free HS-octa did not have the same effect, which suggests that unrestricted, three-dimensional diffusion of free HS-octa does not lead to potent multivalent interactions with viruses.

The HS-octa decoy liposomes described in this study, and decoy receptors in general, may inhibit infection by binding to the conserved virus attachment protein (i.e., HBDs) to prevent virus–host cell interactions. Our study highlights the unique biology of HS-binding viruses. Notably, each of the three viruses responds differently to the HS-octa decoy liposomes. HSV infectivity (Fig. 5) and replication (Fig. 6) are the most sensitive to the HS-octa decoy liposomes. HS binds to two HSV glycoproteins, gB and gC (Laquerre et al., 1998; Mardberg et al., 2001). gB is essential for viral entry and HS-octa binding and blockage is most likely a major factor in HSV neutralization. The inhibition of RSV infectivity (Fig. 3) by the decoy liposomes is on a similar scale to the inhibition of HSV. The two major RSV glycoproteins F and G are required for cell binding and fusion, and both bind to HS (Feldman et al., 1999, 2000). When bound to the decoy liposomes, RSV is unable to either bind or fuse to susceptible target cells. However, RSV replication (Fig. 4) is not blocked by decoy liposomes to the same extent as seen with HSV. This reduction in observed inhibition may relate to how RSV spreads from infected cells by traditional budding and by re-infection, as well as by syncytial formation. This cell-to-cell fusion is mediated by the F glycoprotein on the infected cell's surface and may be a more difficult target for the complex decoy liposome system to block.

The decoy liposomes are least effective in inhibiting hPIV3 infectivity. Our results are in agreement with previous studies that observed only modest reductions of hPIV3 infectivity upon treatment with soluble full-length heparin (Bose and Banerjee, 2002). While our decoy liposomes remain more effective than full-length heparin against hPIV3 (Table 3) they are less effective against hPIV3 than either RSV or HSV. hPIV3 binds to HS through interactions with positively charged amino acids on its fusion glycoprotein. Unlike other HS-binding viruses, hPIV3 utilizes alternative host receptors (Bose and Banerjee, 2002). Furthermore, unlike RSV and HSV, which each bind to HS with two glycoproteins, hPIV3 only binds to HS with one of its three major glycoproteins. Interestingly, about 500-fold higher HS-octa decoy liposomes were required to inhibit hPIV3 than for RSV or HSV, but the fold difference was considerably less for either soluble HS-octa or full length heparin (Table 3).

While the exact biochemical mechanism of either heparin or HS-octa inhibition of these viruses remains to be fully elucidated, these results suggest that each virus reacts to HS differently. The data presented here, along with our previous published work (Hendricks et al., 2013), demonstrate that liposomes can be incorporated with distinct receptors to specifically target pathogens. Decoy liposomes can target multiple pathogens that share a common receptor, and HS-octa decoy liposomes could potentially inhibit additional HS-binding pathogens beyond those tested in this study. The decoy liposome platform has great potential for new therapeutic agents for pathogens that bind to specific host receptors to adhere to or enter a target cell. Decoy liposomes could be functionalized with proteins or synthetic small molecules, as well as glycans, to target practically any pathogen with a defined host receptor. Decoy liposomes may also be useful for treating newly emerged pathogens; if the host attachment receptor can be determined with a high-throughput method such as binding micro-arrays (Hobbie et al., 2013), a decoy liposome could be quickly constructed with off-the-shelf components, such as the ones used here. Further, one could switch the decoy on the liposomes from natural receptors to receptor mimics that have enhanced affinity to the virus. In addition, decoy liposomes could be used to clear glycan binding microbial toxins (Esko and Sharon, 2009) following a diagnosed infection. Glycan- or glycosaminoglycan-based liposomes are not immunogenic or foreign, and therefore should be well tolerated by the body (Dancey et al., 1978).

Multiple issues would need to be addressed to fully explore the utility of decoy liposomes as a therapeutic agent, such as optimizing route of delivery, timing and frequency of administration, assessing clearance, and conducting safety studies *in vivo*. Particularly for HS-octa decoy liposomes, potential adverse effects resulting from thrombin binding will need to be fully explored. By scaling up the production of decoy liposomes for use in appropriately designed *in vivo* studies, such questions can be addressed. We did not observe any overt cytotoxic effects of decoy liposomes on cultured cells in our *in vitro* studies. LDH cytotoxicity assays with unfunctionalized liposomes and sialic acid-functionalized liposomes were negative (unpublished observations). Of note, our sialic acid-based decoy liposomes had efficacy without toxicity in mice (Hendricks et al., 2013). Nevertheless, any potential antagonistic effects on normal cellular processes should be fully explored. Viruses have the potential to develop resistance to therapeutic decoy liposomes. This could be tested *in vitro* by serially passaging decoy liposomes with virus to select for escape variants that

could then be further characterized. Additional design modifications could be incorporated. For example, the HS saccharide chains were specifically modified in this study to promote or remove binding to specific cytokines or growth factors or to remove anti-coagulant activity, and HS-octa also retains the ability to bind to HS-binding cytokines including CXCL-1 (Poluri et al., 2013) and IL-12 (Hasan et al., 1999).

In sum, decoy liposomes could be an efficient means of targeting pathogens in a customizable, highly flexible therapeutic package. In contrast to drug-based antiviral treatments, a receptor-based strategy has the advantage of making the development of resistance after exposure to the agent unlikely given that the virus life cycle is aborted prior to any possibility of viral replication. Liposomal delivery has been commercially successful for a variety of drugs in humans and the fact that the decoy liposome preparations have activity against several different human viruses make this a rational therapeutic agent.

Acknowledgements

This work was supported by Defense Advanced Research Projects Agency, Defense Sciences Office (DSO), Contract No. W911NF-10-1-0268 to R.W.F. and N.I.H. grant AI063106 to D.M.K. We especially thank Melanie Trombly for assistance with the preparation of this manuscript.

Abbreviations

DOPC	1,2-dioleoyl-sn-glycero-3-phosphocholine
DOPE	1,2-dioleoyl-sn-glycero-3-phosphoethanolamine
DOPG	1,2-dioleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)]
HAI	hemagglutination inhibition
HS	heparan sulfate
hPIV3	human parainfluenza virus 3
HSV	herpes simplex virus
HS-octa	HS-octasaccharide
HBDs	HS-binding domains
HMPV	human metapneumovirus
RBCs	red blood cells
RSV	respiratory syncytial virus

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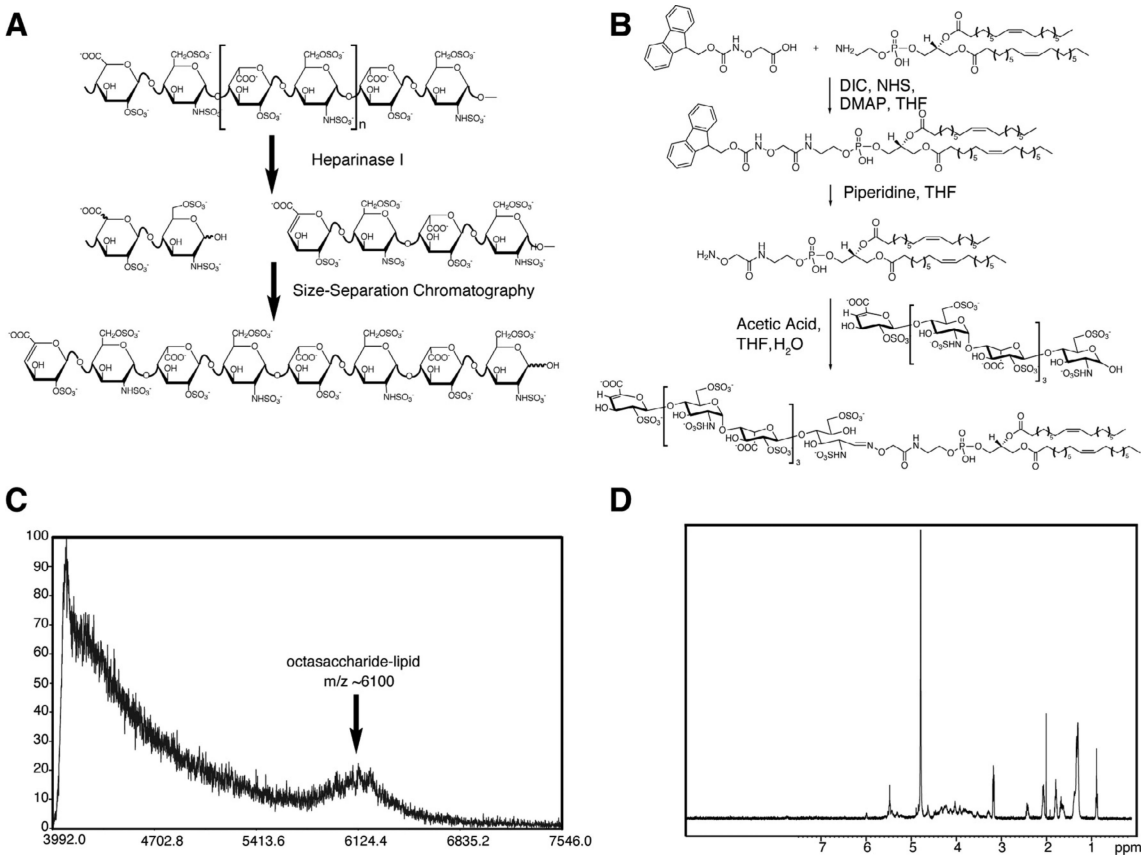


Fig. 1.

Summary of decoy liposome construction. (A) HS-octa synthesis. Full-length heparin was digested with heparinase I, then fast protein liquid chromatography was used to separate digested polysaccharides by size-separation chromatography. (B) The chemical synthesis of HS-octa-DOPE is outlined. Details are provided in Section 2. (C) MALDI-mass spectrum of HS-octa-DOPE lipid conjugate. (D) Proton NMR of HPLC purified HS-octa-DOPE glycolipid to confirm structure of purified product. *Abbreviations:* DIC, N,N′-Diisopropylcarbodiimide; NHS, N-hydroxysuccinimide; DMAP, 4-(dimethylamino)pyridine; THF, tetrahydrofuran.

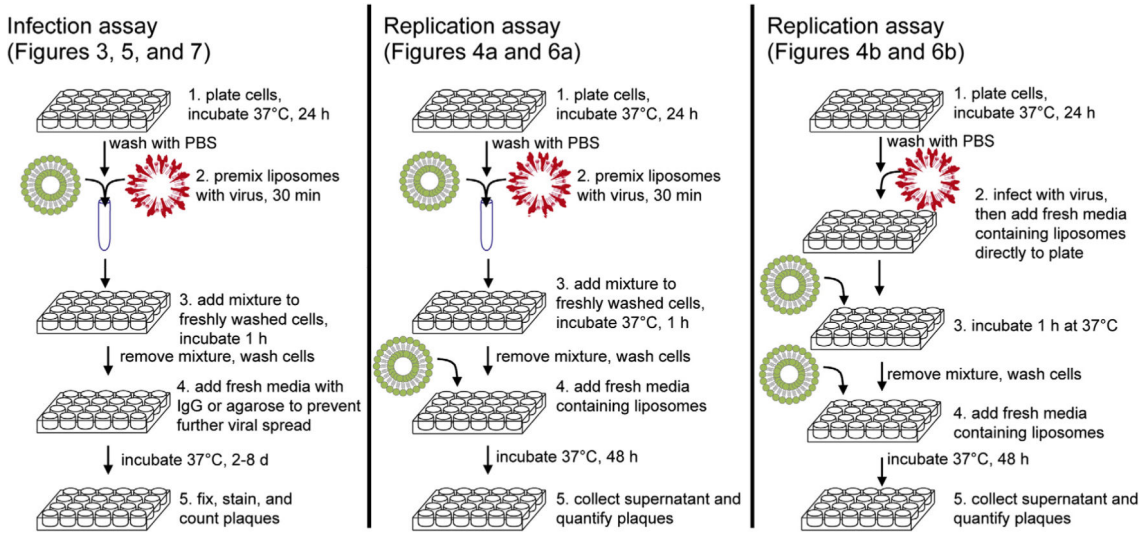


Fig. 2.

Schematic summarizing infection and replication assays with decoy liposomes. The experiments described in Figs. 3–7 are depicted; green represents decoy liposomes and red represents virus. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

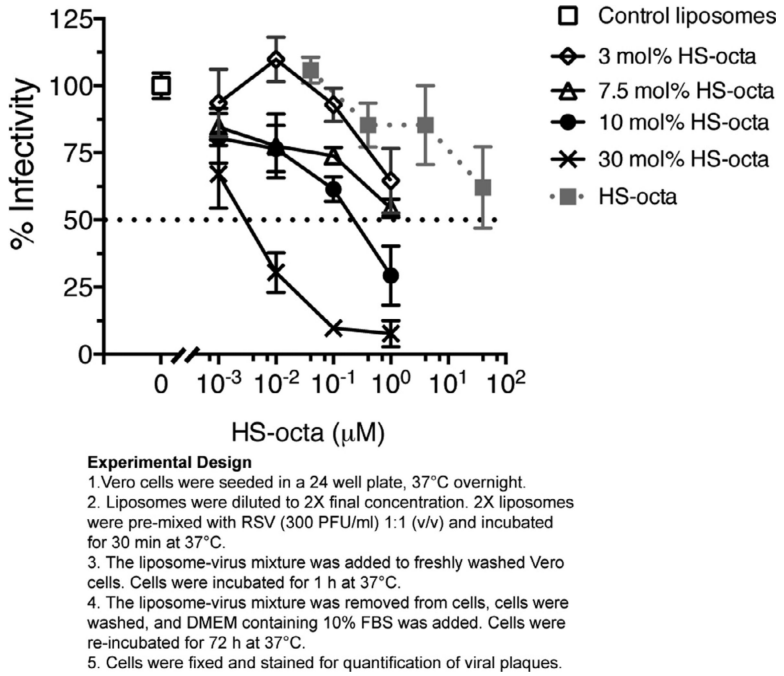


Fig. 3.

HS-octa decoy liposomes inhibit RSV infectivity. RSV was incubated with control liposomes, HS-octa-containing decoy liposomes, or free HS-octa before addition to Vero cells. The PFU/well of virus treated with 0 mol% HS-octa control liposomes is defined as 100% infectivity (open square) and virus treated with different liposome formulations are expressed as a percentage relative to the control. Free HS-octa (gray squares) only significantly inhibited RSV infectivity at 40 μM HS-octa ($P < 0.05$). The 30 mol% HS-octa decoy liposomes (Xs) inhibited RSV to the greatest extent ($P < 0.0001$) at 1 μM HS-octa. The 10 mol% HS-octa decoy liposomes (circles) also significantly inhibited infection ($P < 0.001$) at 1 μM HS-octa. The 7.5 mol% HS-octa decoy liposomes (triangles) or 3 mol% HS-octa decoy liposomes (diamonds) inhibited RSV infection poorly. The dotted line denotes 50% infectivity. The data represent the average ± S.E.M. of three independent experiments.

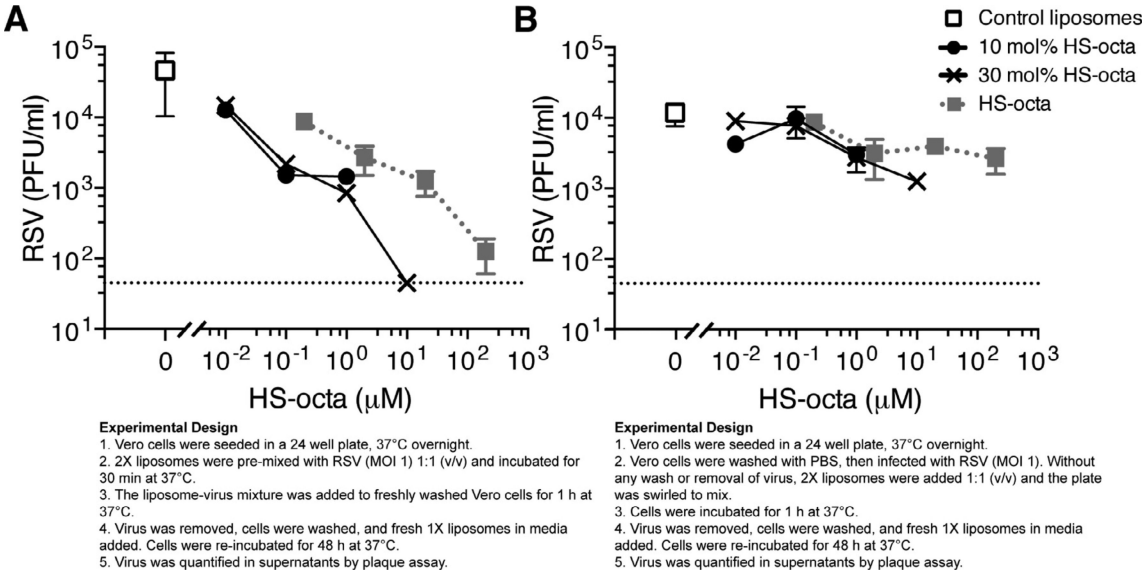
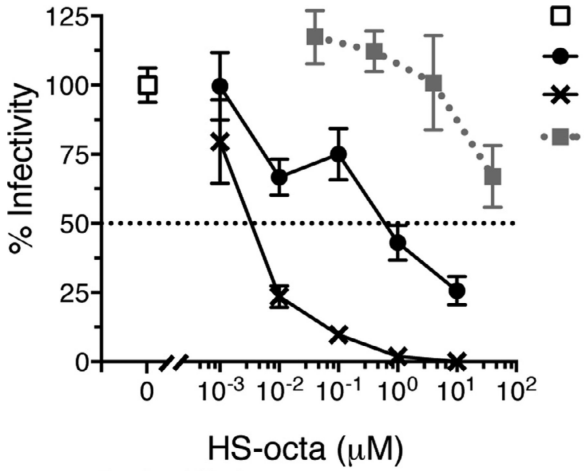


Fig. 4.

RSV replication in Vero cells is blocked by HS-octa decoy liposomes. Control or HS-octa decoy liposomes were pre-complexed with virus (A) or added after viral infection (B) onto confluent monolayers of Vero cells. RSV was used at a final MOI of 1. After initial infection, cells were washed and incubated in the presence of fresh liposomes for 48 h. Viral growth was measured by a PFU assay and plotted as PFU/mL versus concentration of HS-octa present in the solution. (A) 0 mol% HS-octa control liposomes (open square) yielded RSV titers of 27,000 ± 7200 PFU/mL, whereas 10 mol% HS-octa decoy liposomes (circles) reduced RSV titers to 1525 ± 175 PFU/mL and 30 mol% HS-octa decoy liposomes (Xs) reduced titers to 2175 ± 125 PFU/mL at 0.1 μM HS-octa. Free HS-octa (gray squares) was a relatively weak RSV inhibitor. (B) Control liposomes (open squares) yielded RSV titers of 11,800 ± 2100 PFU/mL ($P < 0.05$). Treatment with 30 mol% HS-octa decoy liposomes (Xs) resulted in a significant reduction of RSV with maximal inhibition obtained at 10 μM HS-octa (1250 ± 250 PFU/mL, $P < 0.01$). The dotted line denotes the limit of detection of the assay. The data shown are representative of one of two independent experiments, each with similar results.



Experimental Design

1. Vero cells were seeded in a 96 well plate, 37°C overnight.
2. 2X liposomes were pre-mixed with HSV-1 (300 PFU/ml) 1:1 (v/v) and incubated for 30 min at 37°C.
3. The liposome-virus mixture was added to freshly washed Vero cells. Cells were incubated for 1 h at 37°C.
4. The liposome-virus mixture was removed from cells, cells were washed, and DMEM containing 100 μg/mL pooled human IgG was added. Cells were re-incubated for 48 h at 37°C.
5. Cells were fixed and stained for quantification of viral plaques.

Fig. 5.

HS-octa decoy liposomes inhibit HSV-1 infectivity. HSV-1 was incubated with liposomes or HS-octa before addition to Vero cells. PFU/well of virus treated with control liposomes (0 mol% HS-octa) is defined as 100% infectivity (open square) and virus treated with different liposome formulations is expressed as the percentage of the control. Free HS-octa not incorporated into liposomes (gray squares) only significantly inhibited HSV-1 at the highest dose, 40 μM HS-octa ($P < 0.05$). The 30 mol% HS-octa decoy liposomes (Xs) inhibited HSV-1 infection to the greatest extent, completely blocking HSV-1 infection at 10 μM HS-octa. The 10 mol% HS-octa decoy liposomes (circles) also significantly inhibited infection at 10 μM HS-octa ($P < 0.001$). Free HS-octa was weakly inhibitory. The dotted line denotes 50% infectivity. The data represent the average ± S.E.M. of three experiments.

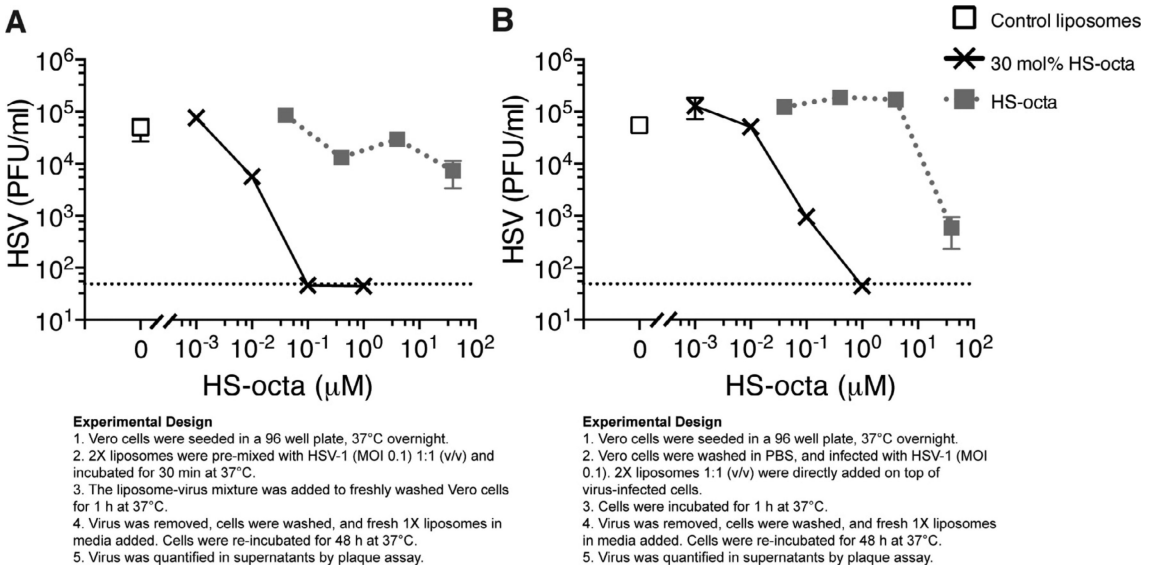
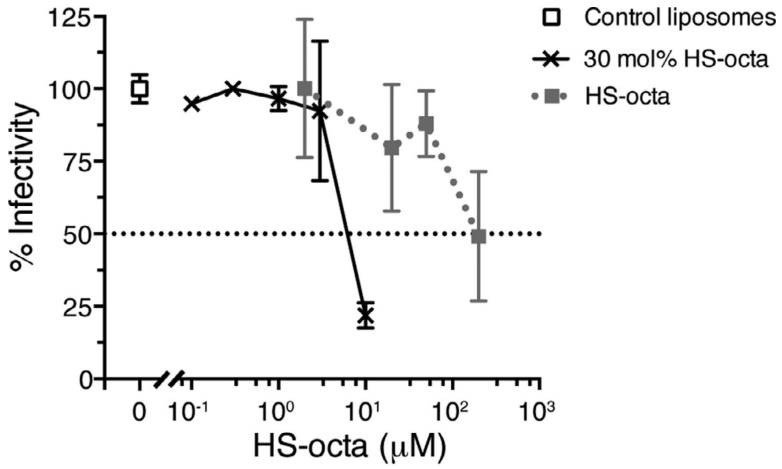


Fig. 6.

HSV-1 replication in Vero cells is blocked by HS-octa decoy liposomes. Control or HS-octa decoy liposomes were pre-complexed with virus (A) or added at the time of infection (B) to confluent monolayers of Vero cells. HSV-1 was used at a final MOI of 0.1. After initial infection, cells were incubated in the presence of fresh liposomes for 48 h. Viral growth was measured by a PFU assay and plotted as PFU/mL versus total HS-octa concentration present in the solution. (A) Control liposomes (0% HS-octa) (open squares), yielded HSV-1 titers of $5 \times 10^6 \pm 1.2 \times 10^4$ PFU/mL. The 30 mol% HS-octa decoy liposomes (Xs) significantly reduced HSV-1 titers to $5.7 \times 10^3 \pm 3.1 \times 10^3$ PFU/mL ($P < 0.01$) at 0.01 μM HS-octa. Free HS-octa (gray squares) inhibited poorly. (B) When added during infection, control liposomes yielded HSV-1 concentrations of $1.3 \times 10^5 \pm 1.2 \times 10^4$ PFU/mL. The 30 mol% HS-octa decoy liposomes reduced concentrations to 950 ± 477 PFU/mL at 0.1 μM HS-octa and below the limit of detection at 1 μM HS-octa. The dotted line denotes the limit of detection of the assay. The data shown are representative of one of two independent experiments, each with similar results.



Experimental Design

1. LLC-MK2 cells were seeded in 6-well plates, 37°C overnight.
2. 2X liposomes were pre-mixed with hPIV3 (50 PFU/ml) 1:1 (v/v) and incubated for 30 min at 37°C.
3. The liposome-virus mixture was added to freshly washed LLC-MK2 cells. Cells were incubated for 1 h at room temperature.
4. The liposome-virus mixture was removed from cells, cells were washed, and media containing 0.8% agarose was added. Cells were re-incubated for 8 d at 37°C.
5. Cells were fixed and stained for quantification of viral plaques.

Fig. 7.

HS-octa decoy liposomes inhibit hPIV3 infectivity. hPIV3 (50 PFU/well) was incubated with control liposomes, HS-octa decoy liposomes, or free HS-octa before the addition to LLC-MK2 cells. PFU/well of virus treated with control liposomes (0 mol% HS-octa) is defined as 100% infectivity (open square), and virus treated with decoy liposomes is expressed as a percentage of the control. The 30 mol% HS-octa decoy liposomes (Xs) significantly inhibited hPIV3 at 10 μM HS-octa ($P < 0.0001$), while free HS-octa (gray squares) was a weak inhibitor. The dotted line denotes 50% infectivity. The data represent the average ± S.E.M. of two experiments.

Table 1

Characterization of HS-octa decoy liposomes.

HS-octa-DOPE (mol%)	DOPC (mol%)	DOPG (mol%)	Radius (nm)[a]	PDI[a]	[Lipid] (μM)	[Liposome] (nM)[b]	Estimated HS-octa:liposome ratio
0	39	30	58.5	0.113	978.2	6.82	0
3	66	0	58.5	0.137	957.5	6.68	4301
7.5	60.5	0	61.7	0.123	909.2	5.71	11,960
10	59	0	62.4	0.144	996.6	6.11	16,261
30	39	0	44.0	0.198	952.7	11.7	24,239

[a]Data acquired by dynamic light scattering (DLS), reported as the average of five measurements, separated by 15 s. PDI, polydispersity index.

[b]Calculated from [Lipid] assuming area per lipid = 0.65 nm^2 and area per liposome = $2(4\pi R^2)$, where R = radius.

Table 2

Decoy liposomes containing HS-octa inhibit hPIV3 hemagglutination.

Treatment	HAI titer[a]	Concentration required for inhibition (μM HS-octa)
Control liposomes	No inhibition[b]	N/A
30 mol% HS-octa liposomes	262,144	4.9
Free HS-octa	32	>240
Heparin	8	66.0

[a] HAI titer is the reciprocal of the furthest dilution that inhibited hemagglutination by hPIV3.

[b] No inhibition was observed at the highest concentration tested.

Table 3

The effect of decoy liposomes and full-length heparin sodium on virus infectivity.

Virus[a]	Decoy liposomes, 30 mol% HS-octa		Heparin sodium		Free HS-octa	
	IC_{50} (μM HS-octa)	r^2	IC_{50} (μM HS)	r^2	IC_{50} (μM HS-octa)	r^2
RSV	0.01	0.65	0.1	0.74	90	0.2
HSV-1	0.01	0.82	1	0.36	14	0.47
hPIV3	5	0.96	68	0.62	100	0.46

[a]Data represents the non-linear regression best fit of the average of at least two independent experiments.