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DESCRIPTION

FIELD OF THE INVENTION

[0001] This invention relates to synthetic nanocarriers that comprise a hydrophobic polyester carrier material and rapamycin that is in a stable, super-saturated amount. Preferably, these synthetic nanocarriers are initially sterile filterable, and, in some embodiments, exhibit *in vivo* efficacy.

SUMMARY OF THE INVENTION

[0002] It has been surprisingly discovered that the concentration of rapamycin in the formulation during synthetic nanocarrier formation, relative to the solubility limit of the rapamycin in said formulation, can have a significant impact on the ability of the resulting synthetic nanocarriers to induce immune tolerance. In addition, how such rapamycin is dispersed through the synthetic nanocarriers can impact whether or not the resulting synthetic nanocarriers are initially sterile filterable. Specifically, compositions of, and related methods, synthetic nanocarriers created under conditions that result in a concentration of rapamycin that exceeds its solubility in the formed nanocarrier suspension are provided. Such synthetic nanocarriers can provide for more durable immune tolerance and be initially sterile filterable.

[0003] In one aspect, a composition comprising synthetic nanocarriers comprising a hydrophobic polyester carrier material and rapamycin, wherein the rapamycin is present in the synthetic nanocarriers in a stable, super-saturated amount and optionally, wherein the synthetic nanocarriers are initially sterile filterable. In a specific embodiment, the rapamycin is present in a stable, super-saturated amount that is less than 50 weight% based on the weight of rapamycin relative to the weight of hydrophobic polyester carrier material is provided.

[0004] In one embodiment of any one of the compositions or methods provided herein, the weights are the recipe weights of the materials that are combined during the formulation of the synthetic nanocarriers. In one embodiment of any one of the compositions or methods provided herein, the weights are the weights of the materials in the resulting synthetic nanocarrier composition.

[0005] In one embodiment of any one of the compositions and methods provided herein, the rapamycin is present in a stable, super-saturated amount that is less than 45 weight%. In one embodiment of any one of the compositions and methods provided herein, the rapamycin is present in a stable, super-saturated amount that is less than 40 weight%. In one embodiment of any one of the compositions and methods provided herein, the rapamycin is present in a stable, super-saturated amount that is less than 35 weight%. In one embodiment of any one of the compositions and methods provided herein, the rapamycin is present in a stable, super-saturated amount that is less than 30 weight%. In one embodiment of any one of the compositions and methods provided herein, the rapamycin is present in a stable, super-saturated amount that is less than 25 weight%. In one embodiment of any one of the compositions and methods provided herein, the rapamycin is present in a stable, super-saturated amount that is less than 20 weight%. In one embodiment of any one of the compositions and methods provided herein, the rapamycin is present in a stable, super-saturated amount that is less than 15 weight%. In one embodiment of any one of the compositions and methods provided herein, the rapamycin is present in a stable, super-saturated amount that is less than 10 weight%. In one embodiment of any one of the compositions and methods provided herein, the rapamycin is present in a stable, super-saturated amount that is greater than 7 weight%.

[0006] In one embodiment of any one of the compositions and methods provided herein, the hydrophobic

polyester carrier material comprises PLA, PLG, PLGA or polycaprolactone. In one embodiment of any one of the compositions and methods provided herein, the hydrophobic polyester carrier material further comprises PLA-PEG, PLGA-PEG or PCL-PEG.

[0007] In one embodiment of any one of the compositions and methods provided herein, the amount of the hydrophobic polyester carrier material in the synthetic nanocarriers is 5-95 weight% hydrophobic polyester carrier material/total solids. In one embodiment of any one of the compositions and methods provided herein, the amount of hydrophobic polyester carrier material in the synthetic nanocarriers is 60-95 weight% hydrophobic polyester carrier material/total solids.

[0008] In one embodiment of any one of the compositions and methods provided herein, the synthetic nanocarriers further comprise a non-ionic surfactant with HLB value less than or equal to 10. In one embodiment of any one of the compositions and methods provided herein, the non-ionic surfactant with HLB value less than or equal to 10 comprises a sorbitan ester, fatty alcohol, fatty acid ester, ethoxylated fatty alcohol, poloxamer, fatty acid, cholesterol, cholesterol derivative, or bile acid or salt. In one embodiment of any one of the compositions and methods provided herein, the non-ionic surfactant with HLB value less than or equal to 10 comprises SPAN 40, SPAN 20, oleyl alcohol, stearyl alcohol, isopropyl palmitate, glycerol monostearate, BRIJ 52, BRIJ 93, Pluronic P-123, Pluronic L-31, palmitic acid, dodecanoic acid, glyceryl tripalmitate or glyceryl trilinoleate. In one embodiment of any one of the compositions and methods provided herein, the non-ionic surfactant with HLB value less than or equal to 10 is SPAN 40.

[0009] In one embodiment of any one of the compositions and methods provided herein, the non-ionic surfactant with HLB value less than or equal to 10 is encapsulated in the synthetic nanocarriers, present on the surface of the synthetic nanocarriers, or both. In one embodiment of any one of the compositions and methods provided herein, the amount of non-ionic surfactant with HLB value less than or equal to 10 is ≥ 0.1 but ≤ 15 weight% non-ionic surfactant with a HLB value less than or equal to 10/hydrophobic polyester carrier material. In one embodiment of any one of the compositions and methods provided herein, the amount of non-ionic surfactant with HLB value less than or equal to 10 is ≥ 1 but ≤ 13 weight% non-ionic surfactant with a HLB value less than or equal to 10/hydrophobic polyester carrier material. In one embodiment of any one of the compositions and methods provided herein, the amount of non-ionic surfactant with HLB value less than or equal to 10 is ≥ 1 but ≤ 9 weight% non-ionic surfactant with a HLB value less than or equal to 10/hydrophobic polyester carrier material.

[0010] In one embodiment of any one of the compositions and methods provided herein, the composition is initially sterile filterable through a 0.22 μm filter.

[0011] In one embodiment of any one of the compositions and methods provided herein, the mean of a particle size distribution obtained using dynamic light scattering of the synthetic nanocarriers is a diameter greater than 120nm. In one embodiment of any one of the compositions and methods provided herein, the diameter is greater than 150nm. In one embodiment of any one of the compositions and methods provided herein, the diameter is greater than 200nm. In one embodiment of any one of the compositions and methods provided herein, the diameter is greater than 250nm. In one embodiment of any one of the compositions and methods provided herein, the diameter is less than 300nm. In one embodiment of any one of the compositions and methods provided herein, the diameter is less than 250nm. In one embodiment of any one of the compositions and methods provided herein, the diameter is less than 200nm.

[0012] In one embodiment of any one of the compositions and methods provided herein, the rapamycin is encapsulated in the synthetic nanocarriers.

[0013] In one embodiment of any one of the compositions and methods provided herein, the composition

further comprises an antigen. In one embodiment of any one of the compositions and methods provided herein, the antigen is admixed with the synthetic nanocarriers in the composition.

[0014] In one embodiment of any one of the compositions and methods provided herein, the composition further comprises a pharmaceutically acceptable carrier.

[0015] In another aspect, a kit comprising any one of the compositions provided herein is provided. In one embodiment of any one of the kits provided, the kit is for use in any one of the methods provided herein. In one embodiment of any one of the kits provided, when the composition does not comprise antigen, the kit further comprises an antigen. In one embodiment of any one of the kits provided, the composition and antigen are contained in separate containers. In one embodiment of any one of the kits provided, the composition and antigen are contained in the same container. In one embodiment of any one of the kits provided, the kit further comprises instructions for use. In one embodiment of any one of the kits provided, the instructions for use include a description of any one of the methods provided herein.

[0016] In another aspect, a method for producing synthetic nanocarriers comprising a hydrophobic polyester carrier material and rapamycin, comprising obtaining or providing the hydrophobic polyester carrier material, obtaining or providing rapamycin in an amount that exceeds the saturation limit of the rapamycin, combining the hydrophobic polyester carrier material and rapamycin, and forming synthetic nanocarriers such that the rapamycin is in a stable, super-saturated amount is provided.

[0017] In another aspect, a method for producing synthetic nanocarriers comprising a hydrophobic polyester carrier material and rapamycin, comprising obtaining or providing the hydrophobic polyester carrier material, obtaining or providing rapamycin in an amount that exceeds the saturation limit of the rapamycin, combining the hydrophobic polyester carrier material and rapamycin, and stabilizing the rapamycin is provided.

[0018] In one embodiment of any one of the methods for producing, the synthetic nanocarriers are formed in the presence of or the rapamycin is stabilized by adding a non-ionic surfactant with HLB values less than or equal to 10.

[0019] In one embodiment of any one of the methods for producing, the synthetic nanocarriers are formed or the rapamycin is stabilized with rapid solvent evaporation of the combined hydrophobic polyester carrier material and rapamycin in the presence of a solvent.

[0020] In one embodiment of any one of the methods for producing, the synthetic nanocarriers are formed or the rapamycin is stabilized with a solid melt process and/or cooling injection molding.

[0021] In one embodiment of any one of the methods for producing, the method further comprises determining the saturation limit of the rapamycin in the hydrophobic polyester carrier material. In one embodiment of any one of the methods for producing, the determining is performed using any one of the formulae provided herein.

[0022] In one embodiment of any one of the methods for producing, the method further comprises filtering the resulting composition. In one embodiment of any one of the methods for producing, the filtering comprises filtering through a 0.22 μm filter.

[0023] In another aspect, a composition produced by any one of the methods for producing provided herein is provided.

[0024] In one embodiment of any one of the compositions or methods provided herein, the rapamycin is present in a super-saturated amount that is at least 1% over the saturation limit of the rapamycin in the

hydrophobic polyester carrier material. In one embodiment of any one of the compositions or methods provided herein, the rapamycin is present in a super-saturated amount that is at least 5% over the saturation limit of the rapamycin in the hydrophobic polyester carrier material. In one embodiment of any one of the compositions or methods provided herein, the rapamycin is present in a super-saturated amount that is at least 10% over the saturation limit of the rapamycin in the hydrophobic polyester carrier material. In one embodiment of any one of the compositions or methods provided herein, the rapamycin is present in a super-saturated amount that is at least 15% over the saturation limit of the rapamycin in the hydrophobic polyester carrier material. In one embodiment of any one of the compositions or methods provided herein, the rapamycin is present in a super-saturated amount that is at least 20% over the saturation limit of the rapamycin in the hydrophobic polyester carrier material. In one embodiment of any one of the compositions or methods provided herein, the rapamycin is present in a super-saturated amount that is at least 25% over the saturation limit of the rapamycin in the hydrophobic polyester carrier material. In one embodiment of any one of the compositions or methods provided herein, the rapamycin is present in a super-saturated amount that is at least 30% over the saturation limit of the rapamycin in the hydrophobic polyester carrier material.

[0025] In another embodiment of any one of the compositions or methods provided herein, the amount of rapamycin exceeds the saturation limit by at least 1%. In another embodiment, the amount of rapamycin exceeds the saturation limit by at least 5%. In another embodiment, the amount of rapamycin exceeds the saturation limit by at least 10%. In another embodiment, the amount of rapamycin exceeds the saturation limit by at least 15%. In another embodiment, the amount of rapamycin exceeds the saturation limit by at least 20%. In another embodiment, the amount of rapamycin exceeds the saturation limit by at least 25%. In another embodiment, the amount of rapamycin exceeds the saturation limit by at least 30%.

[0026] Any one of the formulae provided in the Examples is provided. Any one of the methods provided herein can include a step of determining the concentration of rapamycin using any one of the formulae provided. In any one of the methods provided herein, the formulae is used to determine the saturation limit of rapamycin in a hydrophobic polyester carrier material.

BRIEF DESCRIPTION OF THE DRAWINGS

[0027]

Fig. 1 shows results from an analysis of rapamycin loss and recovery, which exemplify the determination of rapamycin in a super-saturated amount.

Fig. 2 shows the level of IgG with administration of synthetic nanocarriers with a super-saturated amount of rapamycin as described herein.

Fig. 3 shows the level of IgG with administration of synthetic nanocarriers with a super-saturated amount of rapamycin as described herein.

Fig. 4 shows antibody titers using an administration protocol with synthetic nanocarriers prepared with a rapid solvent evaporation method and with a low HLB surfactant as described herein.

Fig. 5 shows the solvent evaporation rate of standard emulsions in various containers. 50 mL beaker (sample 1, diamonds); 125 mm dish (sample 4, squares). The results demonstrate that evaporation can be more rapid in containers with greater surface area.

Fig. 6 shows results demonstrating the ability of co-administered nanocarrier and KLH (keyhole limpet hemocyanin) with rapamycin (RAPA) to induce tolerance. The sera of the mice were analyzed for antibodies to KLH after each KLH challenge.

Fig. 7 shows results demonstrating durable antibody titer reduction with nanocarriers with low HLB surfactants. The acronym "tSIP" refers to the nanocarriers as described.

Fig. 8 shows results demonstrating synthetic nanocarrier+KLH efficacy compared to free rapamycin+KLH in mice. Anti-KLH EC50 at day 35 and 42 antibody titers (after 2 or 3 KLH alone challenges) for mice treated or not with synthetic nanocarrier+KLH (the symbols represent the geometric mean $\pm$ 95% CI). The acronym "NC" refers to the nanocarriers as described.

Fig. 9 shows the treatment protocols for **Example 13**. The acronym "NC" refers to the nanocarriers as described.

Fig. 10 shows synthetic nanocarrier+KLH antigen specificity in mice. Anti-OVA EC50 at Day 65 antibody titers for mice treated or not with synthetic nanocarrier+KLH (the bars represent the geometric mean $\pm$ 95% CI). The acronym "NC" refers to the nanocarriers as described.

DETAILED DESCRIPTION OF THE INVENTION

A. INTRODUCTION

[0028] It has been found that synthetic nanocarriers with rapamycin in super-saturated amounts can promote antigen-specific immune tolerance, even durable antigen-specific immune tolerance. However, in order to produce synthetic nanocarriers with such amounts, in some embodiments, the stable incorporation of the rapamycin is required. Without stable incorporation, in such embodiments, the synthetic nanocarriers may exhibit rapamycin loss and may be difficult to sterile filter through a 0.22 μ m filter. While not wishing to be bound by any particular theory, typically, rapamycin that is not stably incorporated in synthetic nanocarriers can form aggregates that can clog filters used to remove bacteria from synthetic nanocarrier compositions. Such removal is important to result in synthetic nanocarrier compositions with a desirable level of bacteria and, accordingly, result in a composition that is more sterile, a beneficial feature for compositions used for *in vivo* administration. Thus, it is important for super-saturated amounts of rapamycin in synthetic nanocarriers to be stable in order to realize beneficial *in vivo* effects and to be initially sterile filterable.

[0029] Surprisingly, and as demonstrated in the **Examples**, synthetic nanocarrier compositions with stable, super-saturated amounts of rapamycin can be produced and such synthetic nanocarriers can provide durable antigen-specific tolerance in subjects. Methods for producing such synthetic nanocarriers include the use of low HLB surfactants as well as by processes for producing synthetic nanocarriers that, for example, involve rapid solvent evaporation. Results from a number of the **Examples** show that the synthetic nanocarriers produced with such methods can result in synthetic nanocarriers with super-saturated amounts of rapamycin that are initially sterile filterable. Such synthetic nanocarriers have also been shown in the **Examples** to result in synthetic nanocarrier compositions that can exhibit durable antigen-specific tolerance in subjects.

[0030] Accordingly, provided herein are compositions, and related methods, of synthetic nanocarrier compositions comprising rapamycin in stable, super-saturated amounts. Such synthetic nanocarriers can be, preferably, initially sterile filterable. Also, preferably, in some embodiments, the synthetic nanocarrier compositions provided herein not only can promote antigen-specific immune tolerance but can do so at enhanced levels relative to synthetic nanocarriers where the rapamycin is not present in a stable, super-saturated amount.

[0031] The invention will now be described in more detail below.

B. DEFINITIONS

[0032] "Administering" or "administration" or "administer" means providing a material to a subject in a manner that is pharmacologically useful. The term is intended to include causing to be administered in some embodiments. "Causing to be administered" means causing, urging, encouraging, aiding, inducing or directing, directly or indirectly, another party to administer the material.

[0033] "Admixed" refers to mixing one component, such as an antigen, with another, such as synthetic nanocarriers, in a composition. The components that are mixed are made or obtained separately and placed together. Accordingly, the components are not coupled to each other save for possible non-covalent interactions that may occur when placed together in a composition.

[0034] "Amount effective" in the context of a composition or dose for administration to a subject refers to an amount of the composition or dose that produces one or more desired responses in the subject, for example, the generation of an antigen-specific tolerogenic immune response. In some embodiments, the amount effective is a pharmacodynamically effective amount. Therefore, in some embodiments, an amount effective is any amount of a composition or dose provided herein that produces one or more of the desired therapeutic effects and/or immune responses as provided herein. This amount can be for in vitro or in vivo purposes. For in vivo purposes, the amount can be one that a clinician would believe may have a clinical benefit for a subject in need of antigen-specific immune tolerance. Any one of the compositions as provided herein can be in an amount effective.

[0035] Amounts effective can involve reducing the level of an undesired immune response, although in some embodiments, it involves preventing an undesired immune response altogether. Amounts effective can also involve delaying the occurrence of an undesired immune response. An amount that is effective can also be an amount that produces a desired therapeutic endpoint or a desired therapeutic result. In other embodiments, the amounts effective can involve enhancing the level of a desired response, such as a therapeutic endpoint or result. Amounts effective, preferably, result in a tolerogenic immune response in a subject to an antigen. The achievement of any of the foregoing can be monitored by routine methods.

[0036] Amounts effective will depend, of course, on the particular subject being treated; the severity of a condition, disease or disorder; the individual patient parameters including age, physical condition, size and weight; the duration of the treatment; the nature of concurrent therapy (if any); the specific route of administration and like factors within the knowledge and expertise of the health practitioner. These factors are well known to those of ordinary skill in the art and can be addressed with no more than routine experimentation. It is generally preferred that a maximum dose be used, that is, the highest safe dose according to sound medical judgment. It will be understood by those of ordinary skill in the art, however, that a patient may insist upon a lower dose or tolerable dose for medical reasons, psychological reasons or for virtually any other reason.

[0037] In general, doses of the components in the compositions of the invention refer to the amount of the components. Alternatively, the dose can be administered based on the number of synthetic nanocarriers that provide the desired amount.

[0038] "Antigen" means a B cell antigen or T cell antigen. "Type(s) of antigens" means molecules that share the same, or substantially the same, antigenic characteristics. In some embodiments, antigens may be proteins, polypeptides, peptides, lipoproteins, glycolipids, polynucleotides, polysaccharides or are contained

or expressed in cells. In some embodiments, such as when the antigens are not well defined or characterized, the antigens may be contained within a cell or tissue preparation, cell debris, cell exosomes, conditioned media, etc.

[0039] "Antigen-specific" refers to any immune response that results from the presence of the antigen, or portion thereof, or that generates molecules that specifically recognize or bind the antigen. For example, where the immune response is antigen-specific antibody production, antibodies are produced that specifically bind the antigen. As another example, where the immune response is antigen-specific B cell or CD4+ T cell proliferation and/or activity, the proliferation and/or activity results from recognition of the antigen, or portion thereof, alone or in complex with MHC molecules, B cells, etc.

[0040] "Average", as used herein, refers to the arithmetic mean unless otherwise noted.

[0041] "Determining" or "determine" means to ascertain a factual relationship. Determining may be accomplished in a number of ways, including but not limited to performing experiments, or making projections. In embodiments, "determining" or "determine" comprises "causing to be determined." "Causing to be determined" means causing, urging, encouraging, aiding, inducing or directing or acting in coordination with an entity for the entity to ascertain a factual relationship; including directly or indirectly, or expressly or impliedly.

[0042] "Encapsulate" means to enclose at least a portion of a substance within a synthetic nanocarrier. In some embodiments, a substance is enclosed completely within a synthetic nanocarrier. In other embodiments, most or all of a substance that is encapsulated is not exposed to the local environment external to the synthetic nanocarrier. In other embodiments, no more than 50%, 40%, 30%, 20%, 10% or 5% (weight/weight) is exposed to the local environment. Encapsulation is distinct from absorption, which places most or all of a substance on a surface of a synthetic nanocarrier, and leaves the substance exposed to the local environment external to the synthetic nanocarrier. In embodiments of any one of the compositions or methods provided herein the rapamycin and/or non-ionic surfactant with a hydrophilic-lipophilic balance (HLB) value less than or equal to 10 are encapsulated within the synthetic nanocarriers.

[0043] "Hydrophobic polyester carrier material" refers to any pharmaceutically acceptable carrier that can deliver one or more molecules (e.g., rapamycin and a non-ionic surfactant with a HLB value less than or equal to 10) that comprises one or more polyester polymers or units thereof and that has hydrophobic characteristics. Polyester polymers include, but are not limited to, PLA, PLGA, PLG and polycaprolactone. The hydrophobic polyester carrier materials include materials that can form a synthetic nanocarrier or a portion thereof and that can include or be loaded with one or more molecules (e.g., rapamycin and a non-ionic surfactant with a HLB value less than or equal to 10). Generally, carrier materials can allow for delivery of one or more molecules (e.g., rapamycin and a non-ionic surfactant with a HLB value less than or equal to 10) to a target site or target cell, controlled-release of the one or more molecules, and other desired activities. "Hydrophobic" refers to a material that does not substantially participate in hydrogen bonding to water. Such materials are generally non-polar, primarily non-polar, or neutral in charge. A carrier material suitable for the compositions described herein may be selected based on it exhibiting hydrophobicity at some level. Hydrophobic polyester carrier materials, therefore, are those that are hydrophobic overall and may be completely comprised of hydrophobic polyesters or units thereof. In some embodiments, however, the hydrophobic polyester carrier materials are hydrophobic overall and comprise hydrophobic polyesters or units thereof but are in combination with other polymers or units thereof. These other polymers or units thereof may be hydrophobic but are not necessarily so. Such a carrier material may include one or more other polymers or units thereof provided that the matrix of polymers or units thereof is considered hydrophobic.

[0044] "Initially sterile filterable" refers to a composition of synthetic nanocarriers that has not previously been filtered but can be filtered through a filter, such as a 0.22 μm filter, with a throughput of at least 50 grams nanocarrier/ m^2 of filter membrane surface area. In some embodiments of any one of the compositions or methods provided herein, the throughput is determined by taking a 9 mL volume of synthetic nanocarrier suspension and placing it in a 10 mL syringe with any one of the filters as provided herein. The synthetic nanocarrier suspension is then pushed through the filter until no further suspension materials pass through the filter. The throughput can then be calculated based on the material that was pushed through the filter and the remaining suspension material in the syringe. In some embodiments of any one of the compositions or methods provided herein, the initially sterile filterable composition is non-sterile and/or not suitable for in vivo administration (i.e., not substantially pure and comprising soluble components that are less than desirable for administration in vivo). In other embodiments of any one of the compositions or methods provided herein, the initially sterile filterable composition comprises synthetic nanocarriers that have been produced but have not been further processed to produce a clinical grade material. In some embodiments of any one of the compositions or methods provided herein, the initially sterile filterable composition has not previously been filtered but can be filtered through a filter, such as a 0.22 μm filter, with a throughput of at least 60, 70, 80, 90, 100, 120, 130, 140, 160, 200, 250, 300, 350, 500, 750, 1000 or 1500 grams nanocarrier/ m^2 of a filter membrane surface area. The 0.22 μm filter can be any filter with a 0.22 μm pore size. Such filters can be made of a variety of materials, such as polyethylene sulfone, polyvinylidene fluoride, mixed cellulose esters, solvent free cellulose acetate, regenerated cellulose, nylon, etc. Specific examples of filters include Millipore SLGPM33R, Millipore SLGVM33RS, Millipore SLGSM33SS, Sartorius 16534, Sartorius 17764, Sartorius 17845, etc.

[0045] "Maximum dimension of a synthetic nanocarrier" means the largest dimension of a nanocarrier measured along any axis of the synthetic nanocarrier. "Minimum dimension of a synthetic nanocarrier" means the smallest dimension of a synthetic nanocarrier measured along any axis of the synthetic nanocarrier. For example, for a spheroidal synthetic nanocarrier, the maximum and minimum dimension of a synthetic nanocarrier would be substantially identical, and would be the size of its diameter. Similarly, for a cuboidal synthetic nanocarrier, the minimum dimension of a synthetic nanocarrier would be the smallest of its height, width or length, while the maximum dimension of a synthetic nanocarrier would be the largest of its height, width or length. In an embodiment, a minimum dimension of at least 75%, preferably at least 80%, more preferably at least 90%, of the synthetic nanocarriers in a sample, based on the total number of synthetic nanocarriers in the sample, is equal to or greater than 100 nm. In an embodiment, a maximum dimension of at least 75%, preferably at least 80%, more preferably at least 90%, of the synthetic nanocarriers in a sample, based on the total number of synthetic nanocarriers in the sample, is equal to or less than 5 μm . Preferably, a minimum dimension of at least 75%, preferably at least 80%, more preferably at least 90%, of the synthetic nanocarriers in a sample, based on the total number of synthetic nanocarriers in the sample, is greater than 110 nm, more preferably greater than 120 nm, more preferably greater than 130 nm, and more preferably still greater than 150 nm. Aspect ratios of the maximum and minimum dimensions of synthetic nanocarriers may vary depending on the embodiment. For instance, aspect ratios of the maximum to minimum dimensions of the synthetic nanocarriers may vary from 1:1 to 1,000,000:1, preferably from 1:1 to 100,000:1, more preferably from 1:1 to 10,000:1, more preferably from 1:1 to 1000:1, still more preferably from 1:1 to 100:1, and yet more preferably from 1:1 to 10:1.

[0046] Preferably, a maximum dimension of at least 75%, preferably at least 80%, more preferably at least 90%, of the synthetic nanocarriers in a sample, based on the total number of synthetic nanocarriers in the sample is equal to or less than 3 μm , more preferably equal to or less than 2 μm , more preferably equal to or less than 1 μm , more preferably equal to or less than 800 nm, more preferably equal to or less than 600 nm, and more preferably still equal to or less than 500 nm. In preferred embodiments, a minimum dimension of at least 75%, preferably at least 80%, more preferably at least 90%, of the synthetic nanocarriers in a sample, based on the total number of synthetic nanocarriers in the sample, is equal to or greater than 100 nm, more preferably equal to or greater than 120 nm, more preferably equal to or greater than 130 nm, more

preferably equal to or greater than 140 nm, and more preferably still equal to or greater than 150 nm. Measurement of synthetic nanocarrier dimensions (e.g., effective diameter) may be obtained, in some embodiments, by suspending the synthetic nanocarriers in a liquid (usually aqueous) media and using dynamic light scattering (DLS) (e.g., using a Brookhaven ZetaPALS instrument). For example, a suspension of synthetic nanocarriers can be diluted from an aqueous buffer into purified water to achieve a final synthetic nanocarrier suspension concentration of approximately 0.01 to 0.5 mg/mL. The diluted suspension may be prepared directly inside, or transferred to, a suitable cuvette for DLS analysis. The cuvette may then be placed in the DLS, allowed to equilibrate to the controlled temperature, and then scanned for sufficient time to acquire a stable and reproducible distribution based on appropriate inputs for viscosity of the medium and refractive indices of the sample. The effective diameter, or mean of the distribution, is then reported. Determining the effective sizes of high aspect ratio, or non-spheroidal, synthetic nanocarriers may require augmentative techniques, such as electron microscopy, to obtain more accurate measurements. "Dimension" or "size" or "diameter" of synthetic nanocarriers means the mean of a particle size distribution, for example, obtained using dynamic light scattering.

[0047] "Non-ionic surfactant with a HLB value less than or equal to 10", or "low HLB surfactant", as used herein, refers to a non-ionic amphiphilic molecule that has a structure comprising at least one hydrophobic tail and a hydrophilic head or that has hydrophobic groups or regions and hydrophilic groups or regions. The tail portion of surfactants generally consists of a hydrocarbon chain. Surfactants can be classified based on the charge characteristics of the hydrophilic head portion or groups or regions. As used herein, "HLB" refers to the hydrophilic-lipophilic balance or hydrophile-lipophile balance of a surfactant and is a measure of the hydrophilic or lipophilic nature of a surfactant.

[0048] The HLB of any one of surfactants provided herein may be calculated using the Griffin's method or the Davie's method. For example, using the Griffin's method, the HLB of a surfactant is the product of 20 multiplied by the molecular mass of the hydrophilic portion of the surfactant divided by the molecular mass of the entire surfactant. The HLB value is on a scale from 0 to 20, with 0 corresponding to a completely hydrophobic (lipophilic) molecule, and 20 corresponding to a completely hydrophilic (lipophobic) molecule. In some embodiments, the HLB of the surfactant of any one of the compositions or methods provided herein is 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 (e.g., as determined by Griffin's or Davie's method). Examples of such surfactants for use in any one of the compositions and methods provided herein include, without limitation, sorbitan esters, such as SPAN 40, SPAN 20; fatty alcohols, such as oleyl alcohol, stearyl alcohol; fatty acid esters, such as isopropyl palmitate, glycerol monostearate; ethoxylated fatty alcohols, such as BRIJ 52, BRIJ 93; poloxamers, such as Pluronic P-123, Pluronic L-31; fatty acids, such as palmitic acid, dodecanoic acid; triglycerides, such as glyceryl tripalmitate, glyceryl trilinoleate; cholesterol; cholesterol derivatives, such as sodium cholesteryl sulfate, cholesteryl dodecanoate; and bile salts or acids, such as lithocholic acid, sodium lithocholate. Further examples of such surfactants include sorbitan monostearate (SPAN 60), sorbitan tristearate (SPAN 65), sorbitan monooleate (SPAN 80), sorbitan sesquioleate (SPAN 83), sorbitan trioleate (SPAN 85), sorbitan sesquioleate (Arlacel 83), sorbitan dipalmitate, mono and diglycerides of fatty acids, polyoxyethylene sorbitan trioleate (Tween 85), polyoxyethylene sorbitan hexaoleate (G 1086), sorbitan monoisostearate (Montane 70), polyoxyethylene alcohols, polyoxyethylene glycol alkyl ethers, polyoxyethylene (2) oleyl ether (BRIJ 93), polyoxyethylene cetyl ether (BRIJ 52), polyethylene glycol dodecyl ether (BRIJ L4); 1-monotetradecanoyl-rac-glycerol; glyceryl monostearate; glycerol monopalmitate; ethylenediamine tetrakis tetrol (Tetronic 90R4, Tetronic 701), polyoxyethylene (5) nonylphenylether (IGEPAL CA-520), MERPOL A surfactant, MERPOL SE surfactant, and poly(ethylene glycol) sorbitol hexaoleate. Further examples would also be apparent to one of ordinary skill in the art.

[0049] "Pharmaceutically acceptable excipient" or "pharmaceutically acceptable carrier" means a pharmacologically inactive material used together with a pharmacologically active material to formulate the compositions. Pharmaceutically acceptable excipients comprise a variety of materials known in the art,

including but not limited to saccharides (such as glucose, lactose, and the like), preservatives such as antimicrobial agents, reconstitution aids, colorants, saline (such as phosphate buffered saline), and buffers.

[0050] "Providing" means an action or set of actions that an individual performs that supplies a needed item or set of items or methods for the practice of the present invention. The action or set of actions may be taken either directly oneself or indirectly.

[0051] "Rapid solvent evaporation" refers to any solvent evaporation step that can result in synthetic nanocarriers that comprise stable, super-saturated amounts of rapamycin when used as part of a synthetic nanocarrier formulation process. In some embodiments of any one of the methods provided herein, such a step is one where at least 98% of a solvent, such as dichloromethane, is evaporated within 45 minutes of being combined with the hydrophobic polyester carrier material and rapamycin as provided herein. In other embodiments of any one of the methods provided herein, such a step is one where at least 90% of a solvent is evaporated within 30 minutes of being combined with the hydrophobic polyester carrier material and rapamycin as provided herein. In still other embodiments of any one of the methods provided herein, such a step is one where at least 90% of a solvent is evaporated within 15 minutes of being combined with the hydrophobic polyester carrier material and rapamycin as provided herein. Examples of such a step and processes using such a step in the formation of synthetic nanocarriers are provided herein in the **Examples**. Processes that formulate synthetic nanocarriers and that can include one or more steps of solvent evaporation include emulsion processes (such as double emulsion processes), nanoprecipitation, spray drying, rotor systems, and supercritical fluid processes, such as supercritical CO₂. As another example, the process can be one that includes cryomilling. For example, an amount of rapamycin that would result in super-saturation can be dissolved in bulk polymer with solvent, and the solvent evaporated. The resulting material can then be milled down to produce synthetic nanocarriers of the desired size. Still other processes would be known to one of ordinary skill in the art.

[0052] "Saturation limit", as used herein, is a point at which a solvent would not be expected to dissolve or absorb more of a solute. If additional solute is added to the solvent beyond the saturation limit, it may appear in a separate phase (e.g., precipitate). The saturation limit of a solute in a solvent under particular conditions may be calculated based on the solubility of the solute. In some embodiments, the saturation limit can refer to the saturation limit of a solid phase. For example, a solid can be formed by solidification from a uniform mixture of two or more components, but above a certain ratio of materials (i.e., the saturation limit), the ability to form a molecularly-homogeneous phase may be exceeded under normal or equilibrium conditions. Examples of determining the saturation limit of synthetic nanocarriers for rapamycin can be found in the **Examples**. When referring to rapamycin above its saturation limit, the amount of rapamycin (the solute) is above the amount of rapamycin that would be expected to be dispersed in a hydrophobic polyester carrier material or synthetic nanocarrier composition (the solvent). Formulae for determining this saturation limit for rapamycin can be found below in the **Examples**.

[0053] "Solvent" refers to a substance that can dissolve a solute, such as any one or more of the components of the synthetic nanocarriers as provided herein. In some embodiments, the solvents are those that are useful in the formation of synthetic nanocarriers, such as in an emulsion process (e.g., a double emulsion process). Examples of such solvents include dichloromethane, ethyl acetate, chloroform, and propylene carbonate. Examples also include solvent mixtures that are a combination of a low aqueous solubility organic solvent and a water miscible solvent, such as acetone, ethanol, dimethylsulfoxide, dimethylformamide, formamide, etc. Further examples will be known to one of ordinary skill in the art.

[0054] "Subject" means animals, including warm blooded mammals such as humans and primates; avians; domestic household or farm animals such as cats, dogs, sheep, goats, cattle, horses and pigs; laboratory animals such as mice, rats and guinea pigs; fish; reptiles; zoo and wild animals; and the like.

[0055] "Super-saturation" refers to a composition (e.g., a synthetic nanocarrier composition) containing more of a solute (e.g., rapamycin) than can be dissolved within it under equilibrium conditions. In other words, a composition with a super-saturation concentration has a concentration that is beyond the concentration of saturation. In some embodiments, the rapamycin can be above its saturation limit for a hydrophobic polyester carrier material (e.g., alone or in combination with a solvent in the aqueous phase of a formulation process). The amount of rapamycin in a composition may be determined to be super-saturated by any method known in the art, for example, by determining the concentration of the molecule in a composition and comparing that concentration to the predicted saturation concentration (see, for example, the **Examples**, where details of the process, materials and quantities, etc. can be used to calculate the level of super-saturation of rapamycin).

[0056] Other methods for determining whether or not rapamycin is in a super-saturated amount include film casting, X-ray scattering and electron microscopy. Forms of electron microscopy include, but are not limited to, scanning electron microscopy (SEM), transmission electron microscopy (TEM), and cryogenic transmission electron microscopy (cryo-TEM). Super-saturation may also be determined with physical observations during the formation of the synthetic nanocarriers where turbid emulsions containing a hydrophobic polyester carrier material and rapamycin will become more transparent when the volatile organic solvent has almost completely evaporated, and the solution becomes more turbid as the rapamycin in a super-saturated amount condenses into the nanocarrier formulation. As another example, dried synthetic nanocarrier materials can be dispersed into a volatile organic solvent, such as acetone, dichloromethane, ethyl acetate, etc. such that all of the materials are soluble forming a transparent solution and placed onto a glass slide to dry. If super-saturated, the rapamycin separates from the hydrophobic polyester carrier material. Still another method that may be used to determine super-saturation can involve analyzing a portion of a sample by extraction followed by an HPLC method to quantify the amount of rapamycin present. Carrier material may be identified with proton NMR or other orthogonal methods, such as MALDI-MS, etc. and/or quantified once identified with HPLC. One can then determine the saturation limit of the rapamycin in the hydrophobic polyester carrier material experimentally.

[0057] In some embodiments, any one of the compositions provided herein can comprise rapamycin in a super-saturation amount, such as at a super-saturation concentration. In some embodiments, the composition is super-saturated to the point that the rapamycin is at least 1%, 2%, 3%, 4%, 5%, 10%, 15%, 20%, 25%, 30% or more over the saturation limit of the composition. In some embodiments of any one of the compositions provided herein, a super-saturated amount of rapamycin in synthetic nanocarriers is ≥ 6 but ≤ 50 weight% rapamycin/hydrophobic polyester carrier material. In other embodiments of any one of the compositions provided herein, such an amount is ≥ 6 but ≤ 45 , ≥ 6 but ≤ 40 , ≥ 6 but ≤ 35 , ≥ 6 but ≤ 30 , ≥ 6 but ≤ 25 , ≥ 6 but ≤ 20 , ≥ 6 but ≤ 15 weight% rapamycin/hydrophobic polyester carrier material. In other embodiments of any one of the compositions provided herein, such an amount is ≥ 7 but ≤ 45 , ≥ 7 but ≤ 40 , ≥ 7 but ≤ 35 , ≥ 7 but ≤ 30 , ≥ 7 but ≤ 25 , ≥ 7 but ≤ 20 , ≥ 7 but ≤ 15 weight% rapamycin/hydrophobic polyester carrier material. In still other embodiments of any one of the compositions provided herein, such an amount is ≥ 8 but ≤ 24 weight% rapamycin/hydrophobic polyester carrier material. In some embodiments of any one of the compositions provided herein, such an amount is 6, 7, 8, 9, 10, 12, 15, 17, 20, 22, 25, 27, 30, 35, 45 or more weight% rapamycin/hydrophobic polyester carrier material.

[0058] A super-saturated amount of rapamycin is preferably "stable". A super-saturated amount of rapamycin is stable in synthetic nanocarriers if the synthetic nanocarriers retain such an amount when in suspension, in some embodiments. Preferably, synthetic nanocarriers with stable, super-saturated amounts of rapamycin are initially sterile filterable, and initial sterile filterability may serve as a test of the stability of a super-saturated amount of rapamycin in synthetic nanocarriers. In some embodiments, a super-saturated amount of rapamycin in synthetic nanocarriers is said to be stable when the synthetic nanocarriers can be used for beneficial antigen-specific tolerogenic effects when administered *in vivo*. Examples of synthetic

nanocarriers with super-saturated amounts of rapamycin can be found throughout the **Examples**. There are a number of methods by which a super-saturated amounts of rapamycin can be stabilized in synthetic nanocarriers. Such methods include the use of a non-ionic surfactant with HLB value that is less than or equal to 10 in the production of the synthetic nanocarriers, synthetic nanocarrier formation methods that include one or more steps of rapid solvent evaporation, and synthetic nanocarrier formation methods that can include solid melt and/or cooling injection molding.

[0059] "Surfactant" refers to a compound that can lower the surface tension between two liquids or between a liquid and a solid. Surfactants may act as detergents, wetting agents, emulsifiers, foaming agents, and dispersants and can be used in the formation of synthetic nanocarriers as provided herein. In some embodiments, the surfactants are non-ionic surfactants with a HLB value less than or equal to 10.

[0060] "Synthetic nanocarrier(s)" means a discrete object that is not found in nature, and that possesses at least one dimension that is less than or equal to 5 microns in size. As provided herein the synthetic nanocarriers comprise a hydrophobic polyester carrier material. Accordingly, a synthetic nanocarrier can be, but is not limited to, synthetic nanocarriers comprising hydrophobic polyester nanoparticles. Synthetic nanocarriers may be a variety of different shapes, including but not limited to spheroidal, cuboidal, pyramidal, oblong, cylindrical, toroidal, and the like. Synthetic nanocarriers according to the invention comprise one or more surfaces. In embodiments, synthetic nanocarriers may possess an aspect ratio greater than 1:1, 1:1.2, 1:1.5, 1:2, 1:3, 1:5, 1:7, or greater than 1: 10.

[0061] Synthetic nanocarriers according to the invention that have a minimum dimension of equal to or less than about 100 nm, preferably equal to or less than 100 nm, do not comprise a surface with hydroxyl groups that activate complement or alternatively comprise a surface that consists essentially of moieties that are not hydroxyl groups that activate complement. In a preferred embodiment, synthetic nanocarriers according to the invention that have a minimum dimension of equal to or less than about 100 nm, preferably equal to or less than 100 nm, do not comprise a surface that substantially activates complement or alternatively comprise a surface that consists essentially of moieties that do not substantially activate complement. In a more preferred embodiment, synthetic nanocarriers according to the invention that have a minimum dimension of equal to or less than about 100 nm, preferably equal to or less than 100 nm, do not comprise a surface that activates complement or alternatively comprise a surface that consists essentially of moieties that do not activate complement.

[0062] "Total solids" refers to the total weight of all components contained in a composition or suspension of synthetic nanocarriers. In some embodiments of any one of the compositions or methods provided herein, the amount of total solids is determined as the total dry-nanocarrier mass per mL of suspension. This can be determined by a gravimetric method.

[0063] "Weight%" refers to the ratio of one weight to another weight times 100. For example, the weight% can be the ratio of the weight of one component to another times 100 or the ratio of the weight of one component to a total weight of more than one component times 100. Generally, the weight% is measured as an average across a population of synthetic nanocarriers or an average across the synthetic nanocarriers in a composition or suspension.

C. COMPOSITIONS AND RELATED METHODS

[0064] Provided herein are compositions comprising synthetic nanocarriers that comprise a hydrophobic polyester carrier material and rapamycin in a stable, super-saturated amount, and related methods. Such compositions and related methods can result in antigen-specific tolerogenic effects. Thus, the compositions

and related methods provided can be used for any subject in need of antigen-specific immune tolerance. As mentioned above, it was found that delivering a super-saturated amount of rapamycin in synthetic nanocarriers, can provide more durable antigen-specific immune tolerance. However, it has also been discovered that the super-saturated amounts of rapamycin generally are not stable. Stabilizing the rapamycin in synthetic nanocarriers can help retain an appropriate amount in synthetic nanocarriers during formation, which amounts have been shown to be efficacious. Stabilized super-saturated amounts of rapamycin in synthetic nanocarriers also result in synthetic nanocarrier compositions that have the further beneficial effect of being initially sterile filterable.

[0065] Surprisingly, it has been found that certain surfactants, non-ionic surfactants with a hydrophilic-lipophilic balance (HLB) value less than or equal to 10, stabilize rapamycin in synthetic nanocarriers with hydrophobic polyester carrier materials and allow for improved initial sterile filterability. As shown in the **Examples**, increased throughput of synthetic nanocarrier formulations comprising rapamycin, when initially passed through a 0.22 μm filter, was found when surfactants like SPAN 40 were incorporated in the synthetic nanocarrier formulations. As also shown in the **Examples**, a number of such synthetic nanocarriers formulated with a non-ionic surfactant with a hydrophilic-lipophilic balance (HLB) value less than or equal to 10 were also able to provide for durable antigen-specific tolerance in subjects.

[0066] Optimized amounts of the non-ionic surfactant with HLB value less than or equal to 10 in the synthetic nanocarriers as provided herein have also been discovered. In some embodiments of any one of the compositions or methods provided herein, the amount of the non-ionic surfactant with HLB value less than or equal to 10 in the synthetic nanocarriers is ≥ 0.01 but ≤ 20 weight% non-ionic surfactant with a HLB value less than or equal to 10/hydrophobic polyester carrier material. In some embodiments of any one of the compositions or methods provided herein, the amount of the non-ionic surfactant with HLB value less than or equal to 10 in the synthetic nanocarriers is ≥ 0.1 but ≤ 15 , ≥ 0.5 but ≤ 13 , ≥ 1 but ≤ 9 or 10 weight% non-ionic surfactant with a HLB value less than or equal to 10/hydrophobic polyester carrier material. In other embodiments of any one of the compositions or methods provided herein, the amount of the non-ionic surfactant with HLB value less than or equal to 10 in the synthetic nanocarriers is ≥ 0.01 but ≤ 17 , ≥ 0.01 but ≤ 15 , ≥ 0.01 but ≤ 13 , ≥ 0.01 but ≤ 12 , ≥ 0.01 but ≤ 11 , ≥ 0.01 but ≤ 10 , ≥ 0.01 but ≤ 9 , ≥ 0.01 but ≤ 8 , ≥ 0.01 but ≤ 7 , ≥ 0.01 but ≤ 6 , ≥ 0.01 but ≤ 5 , etc. weight% non-ionic surfactant with a HLB value less than or equal to 10/hydrophobic polyester carrier material. In still other embodiments of any one of the compositions or methods provided herein, the amount of the non-ionic surfactant with HLB value less than or equal to 10 in the synthetic nanocarriers is ≥ 0.1 but ≤ 15 , ≥ 0.1 but ≤ 14 , ≥ 0.1 but ≤ 13 , ≥ 0.1 but ≤ 12 , ≥ 0.1 but ≤ 11 , ≥ 0.1 but ≤ 10 , ≥ 0.1 but ≤ 9 , ≥ 0.1 but ≤ 8 , ≥ 0.1 but ≤ 7 , ≥ 0.1 but ≤ 6 , ≥ 0.1 but ≤ 5 , etc. weight% non-ionic surfactant with a HLB value less than or equal to 10/hydrophobic polyester carrier material. In still other embodiments of any one of the compositions or methods provided herein, the amount of the non-ionic surfactant with HLB value less than or equal to 10 in the synthetic nanocarriers is ≥ 0.5 but ≤ 15 , ≥ 0.5 but ≤ 14 , ≥ 0.5 but ≤ 13 , ≥ 0.5 but ≤ 12 , ≥ 0.5 but ≤ 11 , ≥ 0.5 but ≤ 10 , ≥ 0.5 but ≤ 9 , ≥ 0.5 but ≤ 8 , ≥ 0.5 but ≤ 7 , ≥ 0.5 but ≤ 6 , ≥ 0.5 but ≤ 5 , etc. weight% non-ionic surfactant with a HLB value less than or equal to 10/hydrophobic polyester carrier material. In still other embodiments of any one of the compositions or methods provided herein, the amount of the non-ionic surfactant with HLB value less than or equal to 10 in the synthetic nanocarriers is ≥ 1 but ≤ 9 , ≥ 1 but ≤ 8 , ≥ 1 but ≤ 7 , ≥ 1 but ≤ 6 , ≥ 1 but ≤ 5 , etc. weight% non-ionic surfactant with a HLB value less than or equal to 10/hydrophobic polyester carrier material. In still other embodiments of any one of the compositions or methods provided herein, the amount of the non-ionic surfactant with HLB value less than or equal to 10 in the synthetic nanocarriers is ≥ 5 but ≤ 15 , ≥ 5 but ≤ 14 , ≥ 5 but ≤ 13 , ≥ 5 but ≤ 12 , ≥ 5 but ≤ 11 , ≥ 5 but ≤ 10 , ≥ 5 but ≤ 9 , ≥ 5 but ≤ 8 , ≥ 5 but ≤ 7 , ≥ 5 but ≤ 6 , etc. weight% non-ionic surfactant with a HLB value less than or equal to 10/hydrophobic polyester carrier material. In some embodiments of any one of the compositions or methods provided herein, the amount of the non-ionic surfactant with HLB value less than or equal to 10 in the synthetic nanocarriers is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 weight% non-ionic surfactant with a HLB value less than or equal to 10/hydrophobic polyester carrier material. Any one of the HLB values provided herein may be

determined using Griffin's or Davie's method.

[0067] Likewise, it has also been found that methods for producing synthetic nanocarriers that utilize rapid solvent evaporation also can result in synthetic nanocarriers that comprise rapamycin in stable, super-saturated amounts. When such evaporation occurs rapidly, rapamycin is stably incorporated in the synthetic nanocarriers and can lead to beneficial results as described herein. For example, as demonstrated in the **Examples**, synthetic nanocarriers produced with such a process were initially sterile filterable and achieved beneficial immune effects when administered *in vivo*. Specific methods for producing such synthetic nanocarriers are provided in the **Examples**. Other examples of such methods include any process that has one or more solvent evaporation steps. Processes that can include one or more steps of solvent evaporation include emulsion processes (such as double emulsion processes), nanoprecipitation, spray drying and supercritical fluid processes. Still other processes would be apparent to one of ordinary skill in the art.

[0068] Further, the synthetic nanocarriers provided herein may also be produced using a process including solid melt and cooling injection molding. Still other processes would be known to one of ordinary skill in the art.

[0069] As provided herein, the amount of rapamycin in the synthetic nanocarriers can be optimized and stabilized such that the amount results in efficacious results (e.g., durable antigen-specific tolerance) when the synthetic nanocarriers are administered to a subject. In some embodiments of any one of the compositions provided herein, the synthetic nanocarriers that comprise rapamycin in a stable, super-saturated amount comprise ≥ 6 but ≤ 50 weight% rapamycin/hydrophobic polyester carrier material. In some embodiments of any one of the compositions provided herein, the synthetic nanocarriers comprise ≥ 6 but ≤ 45 , ≥ 6 but ≤ 40 , ≥ 6 but ≤ 35 , ≥ 6 but ≤ 30 , ≥ 6 but ≤ 25 , ≥ 6 but ≤ 20 , ≥ 6 but ≤ 15 weight% rapamycin/hydrophobic polyester carrier material. In other embodiments of any one of the compositions provided herein, the synthetic nanocarriers comprise ≥ 7 but ≤ 45 , ≥ 7 but ≤ 40 , ≥ 7 but ≤ 35 , ≥ 7 but ≤ 30 , ≥ 7 but ≤ 25 , ≥ 7 but ≤ 20 , ≥ 7 but ≤ 15 weight% rapamycin/hydrophobic polyester carrier material. In still other embodiments of any one of the compositions provided herein, the synthetic nanocarriers comprise ≥ 8 but ≤ 24 weight% rapamycin/hydrophobic polyester carrier material. In some embodiments of any one of the compositions provided herein, the synthetic nanocarriers comprise 6, 7, 8, 9, 10, 12, 15, 17, 20, 22, 25, 27, 30, 35, 45 or more weight% rapamycin/hydrophobic polyester carrier material.

[0070] Further, optimized amounts of the hydrophobic polyester carrier material in the synthetic nanocarrier compositions have also been determined. Preferably, in some embodiments of any one of the compositions provided herein, the amount of hydrophobic polyester carrier material in the synthetic nanocarrier composition is 5-95 weight% hydrophobic polyester carrier material/total solids. In other embodiments of any one of the compositions provided herein, the amount of hydrophobic polyester carrier material in the synthetic nanocarriers is 10-95, 15-90, 20-90, 25-90, 30-80, 30-70, 30-60, 30-50, etc. weight% hydrophobic polyester carrier material/total solids. In still other embodiments of any one of the compositions provided herein, the amount of hydrophobic polyester carrier materials in the synthetic nanocarriers is 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90 or 95 weight% hydrophobic polyester carrier material/total solids.

[0071] The amounts of components or materials as recited herein for any one of the compositions provided herein can be determined using methods known to those of ordinary skill in the art or otherwise provided herein. For example, amounts of the non-ionic surfactant with a HLB value less than or equal to 10 can be measured by extraction followed by quantitation by an HPLC method. Amounts of hydrophobic polyester carrier material can be determined using HPLC. The determination of such an amount may, in some embodiments, follow the use of proton NMR or other orthogonal methods, such as MALDI-MS, etc. to determine the identity of a hydrophobic polyester carrier material. Similar methods can be used to determine

the amounts of rapamycin in any one of the compositions provided herein. In some embodiments, the amount of rapamycin is determined using HPLC. Further examples of methods for determining amounts of components or materials are as provided elsewhere herein, such as in the **Examples**. For any one of the compositions or methods provided herein the amounts of the components or materials can also be determined based on the recipe weights of a nanocarrier formulation. Accordingly, in some embodiments of any one of the compositions or methods provided herein, the amounts of any one of the components provided herein are those of the components in an aqueous phase during formulation of the synthetic nanocarriers. In some embodiments of any one of the compositions or methods provided herein, the amounts of any one of the components are those of the components in a synthetic nanocarrier composition that is produced and the result of a formulation process.

[0072] The synthetic nanocarriers as provided herein comprise hydrophobic polyester carrier materials. Such materials comprise polyesters, which can include copolymers comprising lactic acid and glycolic acid units, such as poly(lactic acid-co-glycolic acid) and poly(lactide-co-glycolide), collectively referred to herein as "PLGA"; and homopolymers comprising glycolic acid units, referred to herein as "PGA," and lactic acid units, such as poly-L-lactic acid, poly-D-lactic acid, poly-D,L-lactic acid, poly-L-lactide, poly-D-lactide, and poly-D,L-lactide, collectively referred to herein as "PLA." In some embodiments, exemplary polyesters include, for example, polyhydroxyacids; PEG copolymers and copolymers of lactide and glycolide (e.g., PLA-PEG copolymers, PGA-PEG copolymers, PLGA-PEG copolymers, and derivatives thereof. In some embodiments, polyesters include, for example, poly(caprolactone), poly(caprolactone)-PEG copolymers, poly(L-lactide-co-L-lysine), poly(serine ester), poly(4-hydroxy-L-proline ester), poly[α -(4-aminobutyl)-L-glycolic acid], and derivatives thereof.

[0073] In some embodiments, the polyester may be PLGA. PLGA is a biocompatible and biodegradable copolymer of lactic acid and glycolic acid, and various forms of PLGA are characterized by the ratio of lactic acid: glycolic acid. Lactic acid can be L-lactic acid, D-lactic acid, or D,L-lactic acid. The degradation rate of PLGA can be adjusted by altering the lactic acid: glycolic acid ratio. In some embodiments, PLGA to be used in accordance with the present invention is characterized by a lactic acid:glycolic acid ratio of approximately 85:15, approximately 75:25, approximately 60:40, approximately 50:50, approximately 40:60, approximately 25:75, or approximately 15:85.

[0074] The hydrophobic polyester carrier material as provided herein may comprise one or more non-polyester hydrophobic polymers or units thereof and/or polymers or units thereof that are not hydrophobic provided that overall the hydrophobic polyester carrier material is hydrophobic and contains one or more polyesters or units thereof.

[0075] Hydrophobic polyester carrier materials as provided herein may comprise one or more polymers that are a non-methoxy-terminated, pluronic polymer, or a unit thereof. "Non-methoxy-terminated polymer" means a polymer that has at least one terminus that ends with a moiety other than methoxy. In some embodiments, the polymer has at least two termini that ends with a moiety other than methoxy. In other embodiments, the polymer has no termini that ends with methoxy. "Non-methoxy-terminated, pluronic polymer" means a polymer other than a linear pluronic polymer with methoxy at both termini.

[0076] Hydrophobic polyester carrier materials may comprise, in some embodiments, polyhydroxyalkanoates, polyamides, polyethers, polyolefins, polyacrylates, polycarbonates, polystyrene, silicones, fluoropolymers, or a unit thereof. Further examples of polymers that may be comprised in the hydrophobic polyester carrier materials provided herein include polycarbonate, polyamide, or polyether, or unit thereof. In other embodiments, the polymers of the hydrophobic polyester carrier material may comprise poly(ethylene glycol) (PEG), polypropylene glycol, or unit thereof.

[0077] In some embodiments, it is preferred that the hydrophobic polyester carrier material comprises

polymer that is biodegradable. Therefore, in such embodiments, the polymers of the hydrophobic polyester carrier materials may include a polyether, such as poly(ethylene glycol) or polypropylene glycol or unit thereof. Additionally, the polymer may comprise a block-co-polymer of a polyether and a biodegradable polymer such that the polymer is biodegradable. In other embodiments, the polymer does not solely comprise a polyether or unit thereof, such as poly(ethylene glycol) or polypropylene glycol or unit thereof.

[0078] Other examples of polymers suitable for use in the present invention include, but are not limited to polyethylenes, polycarbonates (e.g. poly(1,3-dioxan-2-one)), polyanhydrides (e.g. poly(sebacic anhydride)), polypropylfumerates, polyamides (e.g. polycaprolactam), polyacetals, polyethers, polyesters (e.g., polylactide, polyglycolide, polylactide-co-glycolide, polycaprolactone, polyhydroxyacid (e.g. poly(β -hydroxyalkanoate))), poly(orthoesters), polycyanoacrylates, polyvinyl alcohols, polyurethanes, polyphosphazenes, polyacrylates, polymethacrylates, polyureas, polystyrenes, and polyamines, polylysine, polylysine-PEG copolymers, and poly(ethyleneimine), poly(ethylene imine)-PEG copolymers.

[0079] Still other examples of polymers that may be included in a hydrophobic polyester carrier material include acrylic polymers, for example, acrylic acid and methacrylic acid copolymers, methyl methacrylate copolymers, ethoxyethyl methacrylates, cyanoethyl methacrylate, aminoalkyl methacrylate copolymer, poly(acrylic acid), poly(methacrylic acid), methacrylic acid alkylamide copolymer, poly(methyl methacrylate), poly(methacrylic acid anhydride), methyl methacrylate, polymethacrylate, poly(methyl methacrylate) copolymer, polyacrylamide, aminoalkyl methacrylate copolymer, glycidyl methacrylate copolymers, polycyanoacrylates, and combinations comprising one or more of the foregoing polymers.

[0080] In some embodiments, the polymers of a synthetic nanocarrier associate to form a polymeric matrix. A wide variety of polymers and methods for forming polymeric matrices therefrom are known conventionally. In some embodiments, a synthetic nanocarrier comprising a hydrophobic polyester matrix generates a hydrophobic environment within the synthetic nanocarrier.

[0081] In some embodiments, polymers may be modified with one or more moieties and/or functional groups. A variety of moieties or functional groups can be used in accordance with the present invention. In some embodiments, polymers may be modified with polyethylene glycol (PEG), with a carbohydrate, and/or with acyclic polyacetals derived from polysaccharides (Papisov, 2001, ACS Symposium Series, 786:301). Certain embodiments may be made using the general teachings of US Patent No. 5543158 to Gref et al., or WO publication WO2009/051837 by Von Andrian et al.

[0082] In some embodiments, polymers may be modified with a lipid or fatty acid group. In some embodiments, a fatty acid group may be one or more of butyric, caproic, caprylic, capric, lauric, myristic, palmitic, stearic, arachidic, behenic, or lignoceric acid. In some embodiments, a fatty acid group may be one or more of palmitoleic, oleic, vaccenic, linoleic, alpha-linoleic, gamma-linoleic, arachidonic, gadoleic, arachidonic, eicosapentaenoic, docosahexaenoic, or erucic acid.

[0083] In some embodiments, it is preferred that the polymer is biodegradable. In some embodiments, polymers in accordance with the present invention include polymers which have been approved for use in humans by the U.S. Food and Drug Administration (FDA) under 21 C.F.R. § 177.2600.

[0084] Polymers may be natural or unnatural (synthetic) polymers. Polymers may be homopolymers or copolymers comprising two or more monomers. In terms of sequence, copolymers may be random, block, or comprise a combination of random and block sequences. Typically, polymers in accordance with the present invention are organic polymers.

[0085] In some embodiments, polymers can be linear or branched polymers. In some embodiments,

polymers can be dendrimers. In some embodiments, polymers can be substantially cross-linked to one another. In some embodiments, polymers can be substantially free of crosslinks. In some embodiments, polymers can be used in accordance with the present invention without undergoing a cross-linking step. It is further to be understood that the synthetic nanocarriers may comprise block copolymers, graft copolymers, blends, mixtures, and/or adducts of any of the foregoing and other polymers. Those skilled in the art will recognize that the polymers listed herein represent an exemplary, not comprehensive, list of polymers that can be of use in accordance with the present invention provided they meet the desired criteria.

[0086] The properties of these and other polymers and methods for preparing them are well known in the art (see, for example, U.S. Patents 6,123,727; 5,804,178; 5,770,417; 5,736,372; 5,716,404; 6,095,148; 5,837,752; 5,902,599; 5,696,175; 5,514,378; 5,512,600; 5,399,665; 5,019,379; 5,010,167; 4,806,621; 4,638,045; and 4,946,929; Wang et al., 2001, J. Am. Chem. Soc., 123:9480; Lim et al., 2001, J. Am. Chem. Soc., 123:2460; Langer, 2000, Acc. Chem. Res., 33:94; Langer, 1999, J. Control. Release, 62:7; and Uhrich et al., 1999, Chem. Rev., 99:3181). More generally, a variety of methods for synthesizing certain suitable polymers are described in Concise Encyclopedia of Polymer Science and Polymeric Amines and Ammonium Salts, Ed. by Goethals, Pergamon Press, 1980; Principles of Polymerization by Odian, John Wiley & Sons, Fourth Edition, 2004; Contemporary Polymer Chemistry by Allcock et al., Prentice-Hall, 1981; Deming et al., 1997, Nature, 390:386; and in U.S. Patents 6,506,577, 6,632,922, 6,686,446, and 6,818,732.

[0087] A wide variety of synthetic nanocarriers can be used according to the invention. In some embodiments, synthetic nanocarriers are spheres or spheroids. In some embodiments, synthetic nanocarriers are flat or plate-shaped. In some embodiments, synthetic nanocarriers are cubes or cubic. In some embodiments, synthetic nanocarriers are ovals or ellipses. In some embodiments, synthetic nanocarriers are cylinders, cones, or pyramids.

[0088] In some embodiments, it is desirable to use a population of synthetic nanocarriers that is relatively uniform in terms of size or shape so that each synthetic nanocarrier has similar properties. For example, at least 80%, at least 90%, or at least 95% of the synthetic nanocarriers, based on the total number of synthetic nanocarriers, may have a minimum dimension or maximum dimension that falls within 5%, 10%, or 20% of the average diameter or average dimension of the synthetic nanocarriers.

[0089] Compositions according to the invention can comprise elements in combination with pharmaceutically acceptable excipients, such as preservatives, buffers, saline, or phosphate buffered saline. The compositions may be made using conventional pharmaceutical manufacturing and compounding techniques to arrive at useful dosage forms. In an embodiment, compositions, such as those comprising the synthetic nanocarriers are suspended in sterile saline solution for injection together with a preservative.

[0090] In some embodiments, any component of the synthetic nanocarriers as provided herein may be isolated. Isolated refers to the element being separated from its native environment and present in sufficient quantities to permit its identification or use. This means, for example, the element may be purified as by chromatography or electrophoresis. Isolated elements may be, but need not be, substantially pure. Because an isolated element may be admixed with a pharmaceutically acceptable excipient in a pharmaceutical preparation, the element may comprise only a small percentage by weight of the preparation. The element is nonetheless isolated in that it has been separated from the substances with which it may be associated in living systems, *i.e.*, isolated from other lipids or proteins. Any of the elements provided herein may be isolated and included in the compositions or used in the methods in isolated form.

D. METHODS OF MAKING AND USING THE COMPOSITIONS AND RELATED METHODS

[0091] Synthetic nanocarriers may be prepared using a wide variety of methods known in the art. For example, synthetic nanocarriers can be formed by methods such as nanoprecipitation, flow focusing using fluidic channels, spray drying, single and double emulsion solvent evaporation, solvent extraction, phase separation, milling (including cryomilling), supercritical fluid (such as supercritical carbon dioxide) processing, microemulsion procedures, microfabrication, nanofabrication, sacrificial layers, simple and complex coacervation, and other methods well known to those of ordinary skill in the art. Alternatively or additionally, aqueous and organic solvent syntheses for monodisperse semiconductor, conductive, magnetic, organic, and other nanomaterials have been described (Pellegrino et al., 2005, *Small*, 1:48; Murray et al., 2000, *Ann. Rev. Mat. Sci.*, 30:545; and Trindade et al., 2001, *Chem. Mat.*, 13:3843). Additional methods have been described in the literature (see, e.g., Doubrow, Ed., "Microcapsules and Nanoparticles in Medicine and Pharmacy," CRC Press, Boca Raton, 1992; Mathiowitz et al., 1987, *J. Control. Release*, 5:13; Mathiowitz et al., 1987, *Reactive Polymers*, 6:275; and Mathiowitz et al., 1988, *J. Appl. Polymer Sci.*, 35:755; US Patents 5578325 and 6007845; P. Paolicelli et al., "Surface-modified PLGA-based Nanoparticles that can Efficiently Associate and Deliver Virus-like Particles" *Nanomedicine*. 5(6):843-853 (2010)).

[0092] Various materials may be encapsulated into synthetic nanocarriers as desirable using a variety of methods including but not limited to C. Astete et al., "Synthesis and characterization of PLGA nanoparticles" *J. Biomater. Sci. Polymer Edn*, Vol. 17, No. 3, pp. 247-289 (2006); K. Avgoustakis "Pegylated Poly(Lactide) and Poly(Lactide-Co-Glycolide) Nanoparticles: Preparation, Properties and Possible Applications in Drug Delivery" *Current Drug Delivery* 1:321-333 (2004); C. Reis et al., "Nanoencapsulation I. Methods for preparation of drug-loaded polymeric nanoparticles" *Nanomedicine* 2:8- 21 (2006); P. Paolicelli et al., "Surface-modified PLGA-based Nanoparticles that can Efficiently Associate and Deliver Virus-like Particles" *Nanomedicine*. 5(6):843-853 (2010). Other methods suitable for encapsulating materials into synthetic nanocarriers may be used, including without limitation methods disclosed in United States Patent 6,632,671 to Unger issued October 14, 2003.

[0093] In certain embodiments, synthetic nanocarriers are prepared by a nanoprecipitation process or spray drying. Conditions used in preparing synthetic nanocarriers may be altered to yield particles of a desired size or property (e.g., hydrophobicity, hydrophilicity, external morphology, "stickiness," shape, etc.). The method of preparing the synthetic nanocarriers and the conditions (e.g., solvent, temperature, concentration, air flow rate, etc.) used may depend on the materials to be included in the synthetic nanocarriers and/or the composition of the carrier matrix.

[0094] If synthetic nanocarriers prepared by any of the above methods have a size range outside of the desired range, such synthetic nanocarriers can be sized, for example, using a sieve.

[0095] In embodiments, the synthetic nanocarriers can be combined with an antigen or other composition by admixing in the same vehicle or delivery system.

[0096] Compositions provided herein may comprise inorganic or organic buffers (e.g., sodium or potassium salts of phosphate, carbonate, acetate, or citrate) and pH adjustment agents (e.g., hydrochloric acid, sodium or potassium hydroxide, salts of citrate or acetate, amino acids and their salts) antioxidants (e.g., ascorbic acid, alpha-tocopherol), surfactants (e.g., polysorbate 20, polysorbate 80, polyoxyethylene9-10 nonyl phenol, sodium desoxycholate), solution and/or cryo/lyo stabilizers (e.g., sucrose, lactose, mannitol, trehalose), osmotic adjustment agents (e.g., salts or sugars), antibacterial agents (e.g., benzoic acid, phenol, gentamicin), antifoaming agents (e.g., polydimethylsiloxane), preservatives (e.g., thimerosal, 2-phenoxyethanol, EDTA), polymeric stabilizers and viscosity-adjustment agents (e.g., polyvinylpyrrolidone, poloxamer 488, carboxymethylcellulose) and co-solvents (e.g., glycerol, polyethylene glycol, ethanol).

[0097] Compositions according to the invention may comprise pharmaceutically acceptable excipients. The compositions may be made using conventional pharmaceutical manufacturing and compounding techniques

to arrive at useful dosage forms. Techniques suitable for use in practicing the present invention may be found in Handbook of Industrial Mixing: Science and Practice, Edited by Edward L. Paul, Victor A. Atiemo-Obeng, and Suzanne M. Kresta, 2004 John Wiley & Sons, Inc.; and Pharmaceutics: The Science of Dosage Form Design, 2nd Ed. Edited by M. E. Auten, 2001, Churchill Livingstone. In an embodiment, compositions are suspended in a sterile saline solution for injection together with a preservative.

[0098] It is to be understood that the compositions of the invention can be made in any suitable manner, and the invention is in no way limited to compositions that can be produced using the methods described herein. Selection of an appropriate method of manufacture may require attention to the properties of the particular elements being associated.

[0099] In some embodiments, compositions are manufactured under sterile conditions or are initially or terminally sterilized. This can ensure that resulting compositions are sterile and non-infectious, thus improving safety when compared to non-sterile compositions. This provides a valuable safety measure, especially when subjects receiving the compositions have immune defects, are suffering from infection, and/or are susceptible to infection. In some embodiments, the compositions may be lyophilized and stored in suspension or as lyophilized powder depending on the formulation strategy for extended periods without losing activity.

[0100] Administration may be by a variety of routes, including but not limited to intradermal, intramuscular, subcutaneous, intravenous, and intraperitoneal routes. The compositions referred to herein may be manufactured and prepared for administration using conventional methods.

[0101] The compositions of the invention can be administered in effective amounts, such as the effective amounts described elsewhere herein. Doses of dosage forms may contain varying amounts of elements according to the invention. The amount of elements present in the inventive dosage forms can be varied according to their nature, the therapeutic benefit to be accomplished, and other such parameters. Dose ranging studies can be conducted to establish optimal therapeutic amounts to be present in the dosage form. The elements can be present in the dosage form in an amount effective to generate a desired effect and/or a reduced immune response upon administration to a subject. It may be possible to determine amounts to achieve a desired result using conventional dose ranging studies and techniques in subjects. Inventive dosage forms may be administered at a variety of frequencies. At least one administration of the compositions provided herein may be sufficient to generate a pharmacologically relevant response.

[0102] Another aspect of the disclosure relates to kits. In some embodiments, the kit comprises any one of the compositions provided herein. In some embodiments of any one of the kits provided, the kit comprises synthetic nanocarriers comprising a stable, super-saturated amount rapamycin. In some embodiments of any one of the kits provided, the synthetic nanocarriers are also initially sterile filterable. In other embodiments of any one of the kits provided, the kit comprises a hydrophobic polyester carrier material in any one of the amounts provided herein and rapamycin in any one of the amounts provided herein. In some embodiments of any one of the kits provided, such a kit further comprises a non-ionic surfactant with a hydrophilic-lipophilic balance (HLB) value less than or equal to 10 in any one of the amounts provided herein. In some embodiments of any one of the kits provided, the kit further comprises an antigen. In some embodiments of any one of the kits provided, the compositions or elements thereof can be contained within separate containers or within the same container in the kit. In some embodiments of any one of the kits provided, the container is a vial or an ampoule. In some embodiments of any one of the kits provided, the compositions or elements thereof are contained within a solution separate from the container, such that the compositions or elements may be added to the container at a subsequent time. In some embodiments of any one of the kits provided, the compositions or elements thereof are in lyophilized form each in a separate container or in the same container, such that they may be reconstituted at a subsequent time. In some embodiments of any one

of the kits provided, the kit further comprises instructions for reconstitution, mixing, administration, etc. In some embodiments of any one of the kits provided, the instructions include a description of the methods described herein. Instructions can be in any suitable form, e.g., as a printed insert or a label. In some embodiments of any one of the kits provided herein, the kit further comprises one or more syringes or other device(s) that can deliver synthetic nanocarriers *in vivo* to a subject.

EXAMPLES

Example 1 - Synthetic Nanocarriers with Super-Saturated Amounts of Rapamycin

[0103] Nanocarrier compositions containing the polymers PLGA (3:1 lactide:glycolide, inherent viscosity 0.39 dL/g) and PLA-PEG (5 kDa PEG block, inherent viscosity 0.36 dL/g) as well as the agent rapamycin (RAPA) were synthesized using an oil-in-water emulsion evaporation method. The organic phase was formed by dissolving the polymers and RAPA in dichloromethane. The emulsion was formed by homogenizing the organic phase in an aqueous phase containing the surfactant polyvinylalcohol (PVA). The emulsion was then combined with a larger amount of aqueous buffer and mixed to allow evaporation of the solvent. The RAPA content in the different compositions was varied such that the compositions crossed the RAPA saturation limit of the system as the RAPA content was increased. The RAPA content at the saturation limit for the composition was calculated using the solubility of the RAPA in the aqueous phase and in the dispersed nanocarrier phase. For compositions containing PVA as the primary solute in the aqueous phase, it was found that the RAPA solubility in the aqueous phase is proportional to the PVA concentration such that the RAPA is soluble at a mass ratio of 1:125 to dissolved PVA. For compositions containing the described PLGA and PLA-PEG as the nanocarrier polymers, it was found that the RAPA solubility in the dispersed nanocarrier phase was 7.2% wt/wt. The following formula may be used to calculate the RAPA content at the saturation limit for the composition:

$$RAPA \text{ content} = V(0.008c_{PVA} + 0.072c_{pol})$$

where c_{PVA} is the mass concentration of PVA, c_{pol} is the combined mass concentration of the polymers, and V is the volume of the nanocarrier suspension at the end of evaporation.

Sample ID	Calc. Over Saturation (%)	RAPA Load (%)	Diameter (nm)
1	-50	2.5	143
2	-25	3.8	146
3	1	4.9	147
4	23	4.9	130
5	48	8.1	160
6	73	9.8	189
7	98	12.4	203

[0104] For 1, 2 and 3, a consistent 60% of the RAPA is not recovered, indicating a sub-saturation equilibrium regime between the aqueous and organic phases. For the remaining nanocarriers containing higher amounts of RAPA, a consistent 6.8 mg of RAPA is not recovered. This consistent absolute mass loss indicates that the system is in an oversaturated regime (i.e., is super-saturated in one or more phases).

Example 2 - Synthetic Nanocarriers with Super-Saturated Rapamycin Eliminates or Delays Antibody Development

[0105] Nanocarrier compositions containing the polymers PLGA (3:1 lactide:glycolide, inherent viscosity 0.39 dL/g) and PLA-PEG (5 kDa PEG block, inherent viscosity 0.36 dL/g) as well as the agent RAPA were synthesized using an oil-in-water emulsion evaporation method described in **Example 1**. The RAPA content in the different compositions was varied such that the compositions crossed the RAPA saturation limit of the system as the RAPA content was increased.

Sample ID	Calc. Over Saturation (%)	RAPA Load (%)	Diameter (nm)
1	-50	2.5	143
3	1	4.9	147
8	21	8.5	163
9	48	13.5	159

[0106] To assess the ability of the compositions to induce immune tolerance, mice were intravenously injected three times weekly with co-administered nanocarrier and keyhole limpet hemocyanin (KLH) and then challenged weekly with KLH only. The sera of the mice were then analyzed for antibodies to KLH after KLH challenge. The compositions made in the super-saturated state, and having final RAPA load of 8% or higher, led to absence or delay of antibody development to KLH to a greater extent than the compositions created at or below saturation and having final RAPA load of 5% or lower.

Example 3 - Synthetic Nanocarriers with Super-Saturated Amounts of Rapamycin

[0107] Nanocarrier compositions containing the polymers PLA (inherent viscosity 0.41 dL/g) and PLA-PEG (5 kDa PEG block, inherent viscosity 0.50 dL/g) as well as the agent RAPA were synthesized using the oil-in-water emulsion evaporation method described in **Example 1**. The RAPA content in the different compositions was varied such that the compositions crossed the RAPA saturation limit of the system as the RAPA content was increased. The RAPA content at the saturation limit for the composition was calculated using the method described in **Example 1**. For compositions containing the described PLA and PLA-PEG as the nanocarrier polymers, it was found that the RAPA solubility in the dispersed nanocarrier phase was 8.4% wt/wt. The following formula may be used to calculate the RAPA content at the saturation limit for the composition:

$$RAPA\ content = V(0.008c_{PVA} + 0.084c_{pol})$$

where c_{PVA} is the mass concentration of PVA, c_{pol} is the combined mass concentration of the polymers, and V is the volume of the nanocarrier suspension at the end of evaporation. All nanocarrier lots were filtered through 0.22 μ m filters at the end of formation.

Sample ID	Calc. Over Saturation (%)	RAPA Load (%)	Unwashed Diameter (nm)	Final Diameter (nm)	Filter Throughput (g/m ²)
10	-10	5.4	145	149	>171
11	0	6.2	150	155	>180
12	10	6.1	151	154	>170
13	20	6.1	148	148	80
14	30	6.2	171	151	28
15	40	5.8	202	154	16

[0108] Despite adding increasing amount of RAPA to nanocarriers 12-15, the final RAPA content in the nanocarriers does not increase while filter throughput decreased. This indicates that the compositions were

oversaturated with RAPA, and the excess RAPA was removed during washing and/or filtration.

Example 4 - Synthetic Nanocarriers with Super-Saturated Amounts of Rapamycin Delayed Antibody Production to a Greater Extent than Synthetic Nanocarriers with an Amount of Rapamycin at Saturation

[0109] Nanocarrier compositions containing the polymers PLA (inherent viscosity 0.41 dL/g) and PLA-PEG (5 kDa PEG block, inherent viscosity 0.50 dL/g) as well as the agent rapamycin (RAPA) were synthesized using an oil-in-water emulsion evaporation method. The RAPA content in the different compositions was varied such that one composition was made at the RAPA saturation limit of the system, and one composition was made at 33% oversaturated. In each case half of the material was filtered through a 0.22 μm sterilizing filter, and half remained unfiltered.

Sample ID	Calc. Over Saturation (%)	RAPA Load (%)	Unwashed Diameter (nm)	Final Diameter (nm)	Filter Throughput (g/m^2)
16	0	6.8	126	133	N/A
17	0	5.6	126	131	>148
18	33	9.8	142	149	N/A
19	33	7.9	142	138	37

[0110] To assess the ability of the compositions to induce immune tolerance, mice were intravenously injected three times weekly with co-administered nanocarrier and KLH and then challenged weekly with KLH only. The sera of the mice were then analyzed for antibodies to KLH after three KLH challenges. The composition made in the super-saturated state delayed antibody development to a greater extent than the composition created at saturation. Furthermore the delay and reduction in post-repeated-challenge titer was evident whether the composition had, or had not, been filtered.

Example 5 - More Rapid Solvent Evaporation and Low HLB Surfactant Results in Synthetic Nanocarriers with Super-Saturated Amounts of Rapamycin that are also Initially Sterile Filterable

Materials and Methods

[0111] PLA with an inherent viscosity of 0.41 dL/g was purchased from Evonik Industries AG (Rellinghauser Straße 1-11, Essen Germany), product code 100 DL 4A. PLA-PEG-OMe block co-polymer with a methyl ether terminated PEG block of approximately 5,000 Da and an overall inherent viscosity of 0.50 DL/g was purchased from Evonik Industries AG (Rellinghauser Straße 1-11, Essen Germany), product code 100 DL mPEG 5000 5CE. Rapamycin was purchased from Concord Biotech Limited, 1482-1486 Trasad Road, Dholka 382225, Ahmedabad India. Product code SIROLIMUS. EMPROVE® Polyvinyl Alcohol 4-88 (PVA), USP (85-89% hydrolyzed, viscosity of 3.4-4.6 mPa s) was purchased from EMD Chemicals Inc. (480 South Democrat Road Gibbstown, NJ 08027), product code 1.41350. Cellgro PBS 1X (PBS), was purchased from Corning Incorporated, (One Riverfront Plaza Corning, NY 14831 USA), part number 21-040-CV. Dulbecco's phosphate buffered saline 1X (DPBS) was purchased from Lonza (Muenchensteinerstrasse 38, CH-4002 Basel, Switzerland), product code 17-512Q. Sorbitan monopalmitate was purchased from Croda International (300-A Columbus Circle, Edison, NJ 08837), product code SPAN 40.

[0112] For sample 1, solutions were prepared as follows:

Solution 1: A polymer and rapamycin mixture was prepared by dissolving PLA at 18.75 mg per mL, PLA-PEG-Ome at 6.25 mg per mL, and rapamycin at 4.7 mg per mL of dichloromethane. **Solution 2:** PVA was prepared at 50 mg/mL in 100 mM pH 8 phosphate buffer.

[0113] An O/W emulsion was prepared by combining Solution 1 (1.0 mL) and Solution 2 (3.0 mL) in a small glass pressure tube, vortex mixed for 10 seconds, and was then emulsified by sonication at 30% amplitude for 1 minute with the pressure tube immersed in an ice water bath using a Branson Digital Sonifier 250. The emulsion was then added to an open 500 mL beaker containing DPBS (30 mL). A second O/W emulsion was prepared using the same materials and method as above and then added to the same container containing the first emulsion and DPBS. This was then stirred at room temperature for 2 hours to allow the dichloromethane to evaporate and for the nanocarriers to form. A portion of the nanocarriers was washed by transferring the nanocarrier suspension to a centrifuge tube and centrifuging at 75,600×g and 4 °C for 50 minutes, removing the supernatant, and re-suspended the pellet in DPBS containing 0.25% w/v PVA. The wash procedure was repeated and then the pellet was re-suspended in DPBS containing 0.25% w/v PVA to achieve a nanocarrier suspension having a nominal concentration of 10 mg/mL on a polymer basis. An identical formulation was prepared in a separate 500 mL beaker, processed the same, and pooled together with the first formulation just prior to sterile filtration. The nanocarrier suspension was then filtered using a 33mm diameter 0.22 µm PES membrane syringe filter (Millipore part number SLGP033RB). The filtered nanocarrier suspension was then stored at - 20°C.

[0114] For sample 2, solutions were prepared as follows:

Solution 1: A polymer and rapamycin mixture was prepared by dissolving PLA at 75 mg per mL, PLA-PEG-Ome at 25 mg per mL, and rapamycin at 16 mg per mL in dichloromethane. **Solution 2:** A sorbitan monopalmitate mixture was prepared by dissolving Span 40 at 20 mg/mL in dichloromethane. **Solution 3:** Polyvinyl alcohol was prepared at 50 mg per mL in 100 mM pH 8 phosphate buffer. **Solution 4:** Dichloromethane was filtered using a 0.20µm PTFE membrane syringe filter (VWR part number 28145-491).

[0115] An O/W emulsion was prepared by combining Solution 1 (0.5 mL), Solution 2 (0.125 mL), and Solution 4 (0.375 mL), and Solution 3 (3.0 mL) in a small glass pressure tube, vortex mixed for 10 seconds, and was then emulsified by sonication at 30% amplitude for 1 minute with the pressure tube immersed in an ice water bath using a Branson Digital Sonifier 250. The emulsion was then added to a 50 mL beaker containing DPBS (30 mL). A second O/W emulsion was prepared using the same materials and method as above and then added to the same beaker containing the first emulsion and DPBS. The nanocarrier suspension was then processed in the same way as sample 1.

[0116] For sample 3, solutions were prepared as follows:

Solution 1: A polymer and rapamycin mixture was prepared by dissolving PLA at 37.5 mg per mL, PLA-PEG-Ome at 12.5 mg per mL, and rapamycin at 8 mg per mL in dichloromethane. **Solution 2:** Polyvinyl alcohol was prepared at 75 mg per mL in 100 mM pH 8 phosphate buffer.

[0117] An O/W emulsion was prepared by combining Solution 1 (1 mL) and Solution 2 (3.0 mL) in a small glass pressure tube, vortex mixed for 10 seconds, and was then emulsified by sonication at 30% amplitude for 1 minute with the pressure tube immersed in an ice water bath using a Branson Digital Sonifier 250. An O/W emulsion was formed using the same method as described above for sample 1. After emulsification by sonication, the emulsion was added to a 50 mL beaker containing DPBS (30 mL). A second O/W emulsion was prepared using the same materials and method as above and then added to the same solvent evaporation container. The emulsion was allowed to stir for 2 hours to allow for the organic solvent to evaporate and for the nanocarriers to form. A portion of the nanocarriers was then washed by transferring the nanocarrier suspension to a centrifuge tube and centrifuging at 75,600×g for 50 minutes, removing the

supernatant, and re-suspended the pellet in PBS. The wash procedure was repeated and then the pellet was re-suspended in PBS to achieve a nanocarrier suspension having a nominal concentration of 10 mg/mL on a polymer basis. The nanocarrier suspension was then filtered using a 33mm diameter 0.22 μm PES membrane syringe filter (Millipore part number SLGP033RB). The filtered nanocarrier suspension was then stored at -20°C.

[0118] Nanocarrier size was determined by dynamic light scattering. The amount of rapamycin in the nanocarrier was determined by HPLC analysis. The total dry-nanocarrier mass per mL of suspension was determined by a gravimetric method.

Lot number	SE container	Low HLB surfactant	Surface area of container (cm ²)	Calculated filter throughput (g NP/m ²)	Rapamycin load (%)	Size (nm)	Yield
1	500 mL beaker	None	64	>133	9.84	148	81
2	50 mL beaker	SPAN 40	14	>178	9.32	165	93
3	50 mL beaker	None	14	47	7.38	119	73

[0119] C57BL/6 female mice aged 6 weeks were treated intravenously on d0, 7 and 14 with the nanocarriers mixed with 200 μL of KLH (keyhole limpet hemocyanin) dissolved in PBS at 1 mg/mL. The mice were boosted with 200 μg of KLH on days 21, 28, 35, and 42. Anti-KLH IgG titers were read on days 26, 40, and 47. Results are shown in Fig. 4 and demonstrate that synthetic nanocarriers that comprise stable, super-saturated amounts of rapamycin whether produced with rapid solvent evaporation or with low HLB surfactant significantly reduces antigen-specific antibody production *in vivo*.

Group	Lot ID	SE Container	Low HLB Surfactant	DLS Diameter (nm)	RAPA Load (%)	Filterability (g NP/m ²)
1	PBS	N/A	N/A	N/A	N/A	N/A
6	Sample 1	500 mL beaker	None	148	9.8	>133
12	Sample 2	50 mL beaker	SPAN 40	165	9.3	>178
All	KLH, Sigma #H7017					

Example 6 - More Rapid Solvent Evaporation Leads to Increased Filterability of Synthetic Nanocarriers

Materials and Methods

[0120] For samples 1, 2 and 4, solutions were prepared as follows:

Solution 1: A polymer and rapamycin mixture was prepared by dissolving PLA at 18.8 mg per mL, PLA-PEG-Ome at 6.25 mg per mL, and rapamycin at 4.7 mg per mL in dichloromethane. **Solution 2:** Polyvinyl alcohol was prepared at 50 mg per mL in 100 mM pH 8 phosphate buffer.

[0121] An O/W emulsion was formed in the similar way as described above for **Example 5**, sample 1. After

sonication, the emulsion was added to a beaker containing DPBS (30 mL). A second O/W emulsion was prepared using the same materials and method as above and then added to the same container containing the first emulsion and DPBS. Sample 1 used a 50 mL beaker, sample 2 used a 250 mL beaker, and samples 4 used a 125 mm evaporation dish. For each lot, the double single emulsion was repeated using a new solvent evaporation container of the same size and type, and pooled together with the first after the wash step. The nanocarrier suspension was then processed in the same way as **Example 5**, sample 1.

[0122] Nanocarrier size was determined by dynamic light scattering. The amount of rapamycin in the nanocarrier was determined by HPLC analysis. The total dry-nanocarrier mass per mL of suspension was determined by a gravimetric method. Filter throughput was measured as g/m² of nanocarrier mass through a 0.22 µm filter. In addition, at a nominal 10 mg/mL concentration of polymer added to the formulation, the solution likely passed through only one 33 mm 0.22 µm PES membrane syringe filter having a filter surface area of 4.5 cm. The percent of solvent evaporated from the beakers was calculated by measuring the water loss of the same solvent evaporation containers with 36 mL of the SE buffer over the same time as the formulations (2 hours). The water loss (g), was then subtracted from the measured loss in the formulations to calculate the evaporation of the dichloromethane organic solvent.

Lot number	SE container	SA of container (cm ²)	Calculated throughput (g Np/m ²)	Number of sterile filters required	Rapamycin load	Size (nm)	Yield
1	50 mL beaker	14	121	2	11.37	173	71
2	250 mL beaker	33	>146	1	11.66	156	72
4	125 mm evaporation dish	123	>148	1	11.74	142	73

Example 7 - Method for Determining Super-Saturation

Materials and Methods

[0123] PLA with an inherent viscosity of 0.41 dL/g was purchased from Evonik Industries AG (Rellinghauser Straße 1-11, Essen Germany), product code 100 DL 4A. Rapamycin was purchased from Concord Biotech Limited, 1482-1486 Trasad Road, Dholka 382225, Ahmedabad India. Product code SIROLIMUS.

[0124] Solutions were prepared as follows:

Solution 1: A polymer solution was prepared by dissolving PLA at 100 mg per mL of dichloromethane.

Solution 2: A rapamycin solution was prepared by dissolving rapamycin at 100 mg per mL of dichloromethane.

[0125] Glass microscope slides were cleaned with 70% isopropanol and allowed to dry on a clean, flat surface in a chemical fume hood. Mixture 1 was prepared by mixing 100 µL of Solution 1 with 100 µL of dichloromethane in a glass vial with a solvent resistant screw cap and mixed by vortex mixing. Mixture 2 was prepared using the same method as Mixture 1, with 100 µL of Solution 1, 33.3 µL of Solution 2, and 66.7 µL of dichloromethane. Mixture 3 was prepared using the same method as Mixture 1, using 100 µL of Solution 1 with 66.7 µL of Solution 2, and 33.3 µL of dichloromethane. Next, 50 µL of each mixture was applied to separate locations on the clean glass slides and allowed to dry overnight in the fume hood at room

temperature. A digital image was taken of each dry film and analyzed using image analysis software. Normalized mean intensity increases can show the film becoming opaque above the saturation point.

Mixture	Film						Background				
	Area	Mean Intensity	Standard Deviation	Min	Max	Area	Mean Intensity	Standard Deviation	Min	Max	Normalized Mean Intensity
1	45153	39.9	7.3	18	174	45588	38.6	6	18	123	1.3
2	43444	47.6	7.7	16	148	49698	40.5	5.7	19	95	7.1
3	63995	57.1	35.9	12	232	64441	23.4	4.4	8	85	33.7

Example 8 - Low HLB Surfactant, SM, Increases RAPA Loading and Synthetic Nanocarrier Filterability

[0126] Nanocarrier compositions containing the polymers PLA (inherent viscosity 0.41 dL/g) and PLA-PEG (5 kDa PEG block, inherent viscosity 0.50 dL/g) as well as the hydrophobic drug rapamycin (RAPA) were synthesized, with or without the addition of the low HLB surfactant sorbitan monopalmitate (SM), using the oil-in-water emulsion evaporation method. The organic phase was formed by dissolving the polymers and RAPA in dichloromethane. The emulsion was formed by homogenizing the organic phase in an aqueous phase containing the surfactant PVA using a probe-tip sonicator. The emulsion was then combined with a larger amount of aqueous buffer and mixed to allow dissolution and evaporation of the solvent. The resulting nanocarriers were washed and filtered through a 0.22 μm filter. All compositions contained 100 mg of polymer. The RAPA content in the different compositions was varied.

Sample ID	RAPA Added to Composition (mg)	SM Added to Composition (mg)	Unwashed Diameter (nm)	Final Diameter (nm)	RAPA Load (%)	Filter Throughput (g/m ²)
1	12.2	0	148	148	6.1	80
2	13.3	0	171	151	6.2	28
3	14.3	0	202	154	5.8	16
4	13.6	5	156	161	9.2	>174
5	17	5	168	170	11.8	>184
6	20.4	5	181	179	14.9	77

[0127] For the compositions not containing the surfactant SM (samples 1, 2, and 3), several indications of a limiting ability to fully incorporate RAPA in the nanocarrier composition were observed as increasing amounts of RAPA were added. The increasing difference between the pre- and post-filtration nanocarrier sizes at the higher RAPA formulation levels in the absence of SM were indicative of the presence of larger particulates (individual particles or aggregates) being removed during the washing and/or filtration processes. This was also indicated by the decreased filter throughput before clogging. Finally, adding increasing amounts of RAPA to nanocarrier compositions without SM did not result in increased RAPA loading (for example, sample 1 compared to sample 3), indicating that the additional RAPA was separable from the bulk of the nanocarriers and was removed during the washing and/or filtration steps.

[0128] By contrast, the compositions containing the surfactant SM readily incorporated increased amounts of RAPA. The nanocarrier size was not affected by filtration, and increasing the amount of RAPA added to the composition resulted in increased RAPA loading of the nanocarriers. Some filter throughput reduction was observed at the highest loading level (sample 6), but this may be due to the inherently larger nanocarrier

size. In sum, the incorporation of SM helped to increase RAPA loading and filterability of the synthetic nanocarrier compositions.

Example 9 - SM and Cholesterol Increased RAPA Loading and Filterability

[0129] Nanocarrier compositions were produced using the materials and methods as described in Example 8. Nanocarriers containing polymer and RAPA were produced with varying RAPA load levels. In addition, nanocarriers highly loaded with RAPA were also produced using an excipient, the surfactant SM or cholesterol, in an excipient:RAPA mass ratio of 3.2:1.

Sample ID	Excipient	Diameter (nm)	RAPA Load (%)	Filter Throughput (g/m ²)
7	-	131	5.6	>148
8	-	138	7.9	37
9	SM	165	9.3	>178
10	cholesterol	166	14.3	>180

[0130] The samples of nanocarriers produced in the absence of excipients (samples 7 and 8) demonstrated that the increase in RAPA loading beyond a point of apparent nanocarrier saturation tends to lead to a reduction in filter throughput. The addition of either SM or cholesterol resulted in greater RAPA loading while maintaining stability (samples 9 and 10).

[0131] To assess the ability of the compositions to induce immune tolerance, mice were intravenously injected three times weekly with co-administered nanocarrier and KLH (keyhole limpet hemocyanin) with the same RAPA dose, and then challenged weekly with KLH only. The sera of the mice were then analyzed for antibodies to KLH after each KLH challenge (Fig. 6).

[0132] While all mice receiving RAPA nanocarrier treatment received the same doses of RAPA, the different groups show different degrees of tolerization to KLH. All 5 mice that received the nanocarrier compositions with the lowest load (sample 7) had quantifiable titers of anti-KLH antibodies after the third KLH challenge (at day 40). This group of mice developed reduced titers of anti-KLH antibodies compared to the mice that received PBS only, but exhibited the least tolerization compared to the other nanocarrier groups. Increasing the RAPA load of the nanocarriers in the absence of an excipient (SM or cholesterol) (sample 8) significantly improved tolerization, with only 2 of 5 mice demonstrating quantifiable titers after 3 KLH challenges (at day 40). The composition containing cholesterol as the excipient (sample 10), despite the high RAPA loading of the nanocarriers, resulted in four out of five mice demonstrating significant anti-KLH antibody titers after only two challenges (at day 33). The nanocarrier composition containing SM (sample 9) demonstrated both high-throughput 0.22 μ m filter throughput during production and superior tolerization, with only one out of five mice developing a quantifiable anti-KLH antibody titer after three KLH challenges (at day 40). The results of this study indicate that both excipients (SM and cholesterol) enabled increased nanocarrier loading consistent with tolerance-inducing performance and processing favorability as indicated by filtration throughput. The low HLB surfactant SM provided the properties needed to increase stability of the nanocarriers and demonstrated higher performance.

Example 10- Effects of Low HLB Surfactant on RAPA Load and Filterability

Materials and Methods

[0133] PLA with an inherent viscosity of 0.41 dL/g was purchased from Lakeshore Biomaterials (756 Tom Martin Drive, Birmingham, AL 35211), product code 100 DL 4A. PLA-PEG-OMe block co-polymer with a methyl ether terminated PEG block of approximately 5,000 Da and an overall inherent viscosity of 0.50 DL/g was purchased from Lakeshore Biomaterials (756 Tom Martin Drive, Birmingham, AL 35211), product code 100 DL mPEG 5000 5CE. Rapamycin was purchased from Concord Biotech Limited (1482-1486 Trasad Road, Dholka 382225, Ahmedabad India), product code SIROLIMUS. EMPROVE® Polyvinyl Alcohol 4-88, USP (85-89% hydrolyzed, viscosity of 3.4-4.6 mPa s) was purchased from EMD Chemicals Inc. (480 South Democrat Road Gibbstown, NJ 08027), product code 1.41350. Dulbecco's phosphate buffered saline 1X (DPBS) was purchased from Lonza (Muenchensteinerstrasse 38, CH-4002 Basel, Switzerland), product code 17-512Q. Sorbitan monopalmitate was purchased from Croda International (300-A Columbus Circle, Edison, NJ 08837), product code SPAN 40. Polysorbate 80 was purchased from NOF America Corporation (One North Broadway, Suite 912 White Plains, NY 10601), product code Polysorbate80 (HX2). Sorbitan monolaurate (SPAN 20) was purchased from Alfa Aesar (26 Parkridge Rd Ward Hill, MA 01835), product code L12099. Sorbitan stearate (SPAN 60) was purchased from Sigma-Aldrich (3050 Spruce St. St. Louis, MO 63103), product code S7010. Sorbitan monooleate (SPAN 80) was purchased from Tokyo Chemical Industry Co., Ltd. (9211 North Harborside Street Portland, OR 97203), product code S0060. Octyl β -D-glucopyranoside was purchased from Sigma-Aldrich (3050 Spruce St. St. Louis, MO 63103), product code 08001. Oleyl alcohol was purchased from Alfa Aesar (26 Parkridge Rd Ward Hill, MA 01835), product code A18018. Isopropyl palmitate was purchased from Sigma-Aldrich (3050 Spruce St. St. Louis, MO 63103), product code W515604. Polyethylene glycol hexadecyl ether (BRIJ 52) was purchased from Sigma-Aldrich (3050 Spruce St. St. Louis, MO 63103), product code 388831. Polyethylene glycol oleyl ether (BRIJ 93) was purchased from Sigma-Aldrich (3050 Spruce St. St. Louis, MO 63103), product code 388866. Poly(ethylene glycol)-block-poly(propylene glycol)-*block*-poly(ethylene glycol) (Pluronic L-31) was purchased from Sigma-Aldrich (3050 Spruce St. St. Louis, MO 63103), product code 435406. Poly(ethylene glycol)-block-poly(propylene glycol)-*block*-poly(ethylene glycol) (Pluronic P-123) was purchased from Sigma-Aldrich (3050 Spruce St. St. Louis, MO 63103), product code 435465. Palmitic Acid was purchased from Sigma-Aldrich (3050 Spruce St. St. Louis, MO 63103), product code P0500. DL- α -palmitin was purchased from Sigma-Aldrich (3050 Spruce St. St. Louis, MO 63103), product code M1640. Glyceryl Tripalmitate was purchased from Sigma-Aldrich (3050 Spruce St. St. Louis, MO 63103), product code T5888.

[0134] For Sample 11, solutions were prepared as follows:

Solution 1: A polymer and rapamycin mixture was prepared by dissolving PLA at 75 mg/mL, PLA-PEG-Ome at 25 mg/mL, and rapamycin at 16 mg/mL in dichloromethane. **Solution 2:** A Polysorbate80 mixture was prepared by dissolving Polysorbate80 at 80 mg/mL in dichloromethane. **Solution 3:** Polyvinyl alcohol was prepared at 50 mg/mL in 100 mM pH 8 phosphate buffer.

[0135] An O/W emulsion was prepared by combining Solution 1 (0.5 mL), Solution 2 (0.1 mL), dichloromethane (0.4 mL) and Solution 3 (3.0 mL) in a small glass pressure tube, vortex mixed for 10 seconds, and was then sonicated at 30% amplitude for 1 minute with the pressure tube immersed in an ice water bath, using a Branson Digital Sonifier 250. The emulsion was then added to a 50 mL beaker containing DPBS (30 mL). A second O/W emulsion was prepared using the same materials and method as above and then added to the same container containing the first emulsion and DPBS. This was then stirred at room temperature for 2 hours to allow the dichloromethane to evaporate and for the nanocarriers to form. A portion of the nanocarriers was washed by transferring the nanocarrier suspension to a centrifuge tube and centrifuging at 75,600 $\times$ g and 4 °C for 50 minutes, removing the supernatant, and re-suspended the pellet in DPBS containing 0.25% w/v PVA. The wash procedure was repeated and then the pellet was re-suspended in DPBS containing 0.25% w/v PVA to achieve a nanocarrier suspension having a nominal concentration of 10 mg/mL on a polymer basis. The nanocarrier suspension was then filtered using a 0.22 μ m PES membrane syringe filter (Millipore part number SLGP033RB). The filtered nanocarrier suspension was then

stored at -20°C.

[0136] For samples 12-25, solutions were prepared as follows:

Solution 1: A polymer and rapamycin mixture was prepared by dissolving PLA at 75 mg/mL, PLA-PEG-Ome at 25 mg/mL, and rapamycin at 16 mg/mL in dichloromethane. **Solution 2:** The HLB mixture was prepared by dissolving the HLB surfactant at 5.0 mg/mL in dichloromethane. HLB surfactants include SPAN 20, SPAN 40, SPAN 60, SPAN 80, octyl β -D-glucopyranoside, oleyl acid, isopropyl palmitate, BRIJ 52, BRIJ 93, Pluronic L-31, Pluronic P-123, palmitic acid, DL- α -palmitin, and glyceryl tripalmitate. **Solution 3:** Polyvinyl alcohol was prepared at 62.5 mg/mL in 100 mM pH 8 phosphate buffer.

[0137] An O/W emulsion was prepared by combining Solution 1 (0.5 mL), Solution 2 (0.5 mL), and Solution 3 (3.0 mL) in a small glass pressure tube, vortex mixed for 10 seconds, and was then sonicated at 30% amplitude for 1 minute with the pressure tube immersed in an ice water bath using a Branson Digital Sonifier 250. The emulsion was then added to a 50 mL beaker containing DPBS (30 mL). A second O/W emulsion was prepared using the same materials and method as above and then added to the same beaker containing the first emulsion and DPBS. This was then stirred at room temperature for 2 hours to allow the dichloromethane to evaporate and for the nanocarriers to form. A portion of the nanocarriers was washed by transferring the nanocarrier suspension to a centrifuge tube and centrifuging at 75,600 $\times$ g and 4 °C for 50 minutes, removing the supernatant, and re-suspended the pellet in DPBS containing 0.25% w/v PVA. The wash procedure was repeated and then the pellet was re-suspended in DPBS containing 0.25% w/v PVA to achieve a nanocarrier suspension having a nominal concentration of 10 mg/mL on a polymer basis. The nanocarrier suspension was then filtered using a 0.22 μ m PES membrane syringe filter (Millipore part number SLGP033RB). The filtered nanocarrier suspension was then stored at - 20°C.

Sample	Organic Phase Surfactant	HLB of Surfactant	Size (nm)	Filtration	Number of Filters	Calculated g NP/m ²	Yield (%)	Rapamycin Load (%)
11	Polysorbate 80	15	184	Millex 0.22 μ m	>1	22	91	9.7
12	SPAN 20	8.6	148	Millex 0.22 μ m	1	>144	71	11.2
13	SPAN 40	6.7	149	Millex 0.22 μ m	1	>154	77	11.2
14	SPAN 60	4.7	151	Millex 0.22 μ m	1	>154	77	11.0
15	SPAN 80	4.3	144	Millex 0.22 μ m	1	>169	85	11.1
16	octyl β -D-glucopyranoside	12	127	Millex 0.22 μ m	3	47	64	6.7
17	oleyl alcohol	1.3	165	Millex 0.22 μ m	1	>157	78	12.5
18	isopropyl palmitate	2.9	171	Millex 0.22 μ m	1	>144	71	10.9
19	Brij 52	5	182	Millex 0.22 μ m	1	>138	77	11.2
20	Brij 93	4	174	Millex 0.22 μ m	1	>158	79	11.9
21	Pluronic L-31	1-7	169	Millex 0.22 μ m	4	31	70	8.5
22	Pluronic P-123	7-9	162	Millex	1	>145	72	10.7

Sample	Organic Phase Surfactant	HLB of Surfactant	Size (nm)	Filtration	Number of Filters	Calculated g NP/m ²	Yield (%)	Rapamycin Load (%)
				0.22 µm				
23	Palmitic Acid	3.2	132	Millex 0.22 µm	1	>141	71	1.0
24	DL-α-palmitin	7.2	153	Millex 0.22 µm	3	51	68	7.4
25	Glyceryl Tripalmitate	4.3	168	Millex 0.22 µm	1	>146	73	10.0

[0138] The HLB for most of the low HLB surfactants was determined using publicly available information. For DL-α-Palmitin, the HLB was calculated using the following formula: $Mw = 330.5 \text{ g/mol}$, hydrophilic portion = 119.0 g/mol ; $HLB = 119.0 / 330.5 * 100 / 5 = 7.2$. For Glyceryl Palmitate, the HLB was calculated using the following formula: $Mw = 807.3 \text{ g/mol}$, hydrophilic portion = 173.0 g/mol ; $HLB = 173.0 / 807.3 * 100 / 5 = 4.3$. For Isopropyl Palmitate, the HLB was calculated using the following formula: $Mw = 298.5 \text{ g/mol}$, hydrophilic portion = 44.0 g/mol ; $HLB = 44.0 / 298.5 * 100 / 5 = 2.9$. For Oleyl Alcohol, the HLB was calculated using the following formula: $Mw = 268.5 \text{ g/mol}$, hydrophilic portion = 17.0 g/mol ; $HLB = 17.0 / 268.5 * 100 / 5 = 1.3$. In addition, the load of low HLB surfactant was measured by extraction followed by quantitation by an HPLC method.

[0139] Prior to injection into animals, the bulk nanocarrier suspension was thawed in a room temperature water bath for 30 minutes. The nanocarriers were diluted with DPBS to reach a desired concentration of 278 µg/mL rapamycin. C57BL/6 female mice aged 6 weeks were treated intravenously on d0, 7, and 14 with nanocarriers (1.17 mL) mixed with 130 µL of 10x KLH (Keyhole limpet hemocyanin). The mice were boosted with 200 µg of KLH on days 21, 28, 35, and 42. Anti-KLH IgG titers (measured by ELISA) were read on days 40, 47, and 61. The results demonstrate that low HLB surfactant can result in substantial rapamycin loads and synthetic nanocarrier filterability. In addition, all of the nanocarriers with low HLB surfactants as shown in **Fig. 7** resulted in reduced antibody titers for at least 40 and 47 days.

Example 11 - Effect of Low HLB Surfactant on Synthetic Nanocarrier Filterability

Materials and Methods

[0140] PLA-PEG-OMe block co-polymer with a methyl ether terminated PEG block of approximately 5,000 Da and an overall inherent viscosity of 0.50 DL/g was purchased from Evonik Industries (Rellinghauser Straße 1-11 45128 Essen, Germany), product code 100 DL mPEG 5000 5CE. PLA with an inherent viscosity of 0.41 dL/g was purchased from Evonik Industries (Rellinghauser Straße 1-11 45128 Essen Germany), product code 100 DL 4A. Rapamycin was purchased from Concord Biotech Limited, 1482-1486 Trasad Road, Dholka 382225, Ahmedabad India. Product code SIROLIMUS. Sorbitan monopalmitate was purchased from Croda (315 Cherry Lane New Castle Delaware 19720), product code SPAN 40. Dichloromethane was purchased from Spectrum (14422 S San Pedro Gardena CA, 90248-2027). Part number M1266. EMPROVE® Polyvinyl Alcohol 4-88, USP (85-89% hydrolyzed, viscosity of 3.4-4.6 mPa·s) was purchased from EMD Chemicals Inc. (480 South Democrat Road Gibbstown, NJ 08027), product code 1.41350. Dulbecco's Phosphate Buffered Saline, 1X, 0.0095 M (PO₄), without calcium and magnesium, was purchased from BioWhittaker (8316 West Route 24 Mapleton, IL 61547), part number #12001, product code

Lonza DPBS. Emulsification was carried out using a Branson Digital Sonifier 250 with a 1/8" tapered tip titanium probe.

[0141] Solutions were prepared as follows:

Solution 1: A polymer mixture was prepared by dissolving PLA-PEG-OMe (100 DL mPEG 5000 5CE) at 50 mg per 1 mL and PLA (100 DL 4A) at 150 mg per mL in dichloromethane. **Solution 2:** Rapamycin was dissolved at 160 mg per 1 mL in dichloromethane. **Solution 5:** Sorbitan monopalmitate (SPAN 40) was dissolved at 50 mg per 1 mL in dichloromethane. **Solution 6:** Dichloromethane was sterile filtered using a 0.2 µm PTFE membrane syringe filter (VWR part number 28145-491). **Solution 7:** A polyvinyl alcohol solution was prepared by dissolving polyvinyl alcohol (EMPROVE® Polyvinyl Alcohol 4-88) at 75 mg per 1 mL in 100 mM pH 8 phosphate buffer. **Solution 8:** A polyvinyl alcohol and Dulbecco's phosphate buffered saline, 1X, 0.0095 M (PO4) mixture was prepared by dissolving polyvinyl alcohol (EMPROVE® Polyvinyl Alcohol 4-88) at 2.5 mg per 1 mL in Dulbecco's phosphate buffered saline, 1X, 0.0095 M (PO4) (Lonza DPBS).

[0142] For sample 26, an O/W emulsion was prepared by combining Solution 1 (0.5 mL), Solution 2 (0.1 mL), Solution 5 (0.1 mL), and Solution 6 (0.30 mL) in a small glass pressure tube. The solution was mixed by repeat pipetting. Next, Solution 7 (3.0 mL) was added, and the formulation was vortex mixed for ten seconds. The formulation was then sonicated with the pressure tube immersed in an ice bath for 1 minute at 30% amplitude. The emulsion was then added to an open 50 mL beaker containing Lonza DPBS (30 mL). This was then stirred at room temperature for 2 hours to allow the dichloromethane to evaporate and for the nanocarriers to form. A portion of the nanocarriers were washed by transferring the nanocarrier suspension to a centrifuge tube and centrifuging at 75,600×g and 4 °C for 50 minutes, removing the supernatant, and re-suspending the pellet in Solution 8. The wash procedure was repeated and then the pellet was re-suspended in Solution 8 to achieve a nanocarrier suspension having a nominal concentration of 10 mg per mL on a polymer basis. The nanocarrier formulation was filtered using a 0.22 µm PES membrane syringe filter (Millex part number SLGP033RS). The mass of the nanocarrier solution filter throughput was measured. The filtered nanocarrier solution was then stored at -20°C.

[0143] For sample 27, an O/W emulsion was prepared by combining Solution 1 (0.5 mL), Solution 2 (0.1 mL), and Solution 6 (0.40 mL) in a small glass pressure tube. The solution was mixed by repeat pipetting. Next, Solution 7 (3.0 mL) was added, and the formulation was vortex mixed for ten seconds. The formulation was then sonicated with the pressure tube immersed in an ice bath for 1 minute at 30% amplitude. The emulsion was then added to a 50 mL open beaker containing Lonza DPBS (30 mL). This was then stirred at room temperature for 2 hours to allow the dichloromethane to evaporate and for the nanocarriers to form. A portion of the nanocarriers were washed by transferring the nanocarrier suspension to a centrifuge tube and centrifuging at 75,600×g and 4 °C for 50 minutes, removing the supernatant, and re-suspending the pellet in Solution 8. The wash procedure was repeated and then the pellet was re-suspended in Solution 8 to achieve a nanocarrier suspension having a nominal concentration of 10 mg per mL on a polymer basis. The nanocarrier formulation was filtered using a 0.22 µm PES membrane syringe filter (Millex part number SLGP033RS). The mass of the nanocarrier solution filter throughput was measured. The filtered nanocarrier solution was then stored at -20°C.

[0144] Nanocarrier size was determined by dynamic light scattering. The amount of rapamycin in the nanocarrier was determined by HPLC analysis. The total dry-nanocarrier mass per mL of suspension was determined by a gravimetric method. The filterability was evaluated by the amount of filtrate that passed through the first filter.

[0145] The data show that for rapamycin, the incorporation of SPAN 40 in the synthetic nanocarriers resulted in an increase in filterability of the synthetic nanocarrier compositions.

Nanocarrier ID	Rapamycin	Low HLB Surfactant	Effective Diameter (nm)	Rapamycin Content (% w/w)	Nanocarrier Yield (%)	0.22µm Filter Throughput (g/m ²)
26	Rapamycin	SPAN 40	179	17.19	80	98
27	Rapamycin	None	226	17.56	75	10

Example 12 - SPAN 40 Greatly Increases Filterability of Synthetic Nanocarriers Comprising Polyester Polymers

Materials and Methods

[0146] PLA (100 DL 4A), with an inherent viscosity of 0.41 dL/g was purchased from Evonik Industries AG (Rellinghauser Straße 1-11, Essen Germany), product code 100 DL 4A. PLA-PEG-OMe block co-polymer with a methyl ether terminated PEG block of approximately 5,000 Da and an overall inherent viscosity of 0.50 DL/g was purchased from Evonik Industries AG (Rellinghauser Straße 1-11, Essen Germany), product code 100 DL mPEG 5000 5CE. Rapamycin was purchased from Concord Biotech Limited (1482-1486 Trasad Road, Dholka 382225, Ahmedabad India), product code SIROLIMUS. EMPROVE® Polyvinyl Alcohol 4-88 (PVA), USP (85-89% hydrolyzed, viscosity of 3.4-4.6 mPa·s) was purchased from EMD Chemicals Inc. (480 South Democrat Road Gibbstown, NJ 08027), product code 1.41350. Dulbecco's phosphate buffered saline 1X (DPBS) was purchased from Lonza (Muenchensteinerstrasse 38, CH-4002 Basel, Switzerland), product code 17-512Q. Sorbitan monopalmitate (SPAN 40), was purchased from Croda International (300-A Columbus Circle, Edison, NJ 08837), product code Span 40. PLGA (5050 DLG 2.5A), with approximately 54% by weight lactide and 46% by weight glycolide, and an inherent viscosity of 0.24 dL/g was purchased from Evonik Industries AG (Rellinghauser Straße 1-11, Essen Germany), product code 5050 DLG2. 5A. PLGA (7525 DLG 4A), with approximately 73% by weight lactide and 27% by weight glycolide, and an inherent viscosity of 0.39 dL/g was purchased from Evonik Industries AG (Rellinghauser Straße 1-11, Essen Germany), product code 7525 DLG 4A. Polycaprolactone (PCL), average Mw 14,000 Da and Mn of 10,000 Da, was purchased from Sigma-Aldrich (3050 Spruce St. St. Louis, MO 63103), product code 440752.

[0147] For samples 1, 3, 5 and 7, solutions were prepared as follows:

Solution 1: PLA-PEG-Ome at 50 mg per mL, Span 40 at 10 mg per mL and rapamycin at 32 mg per mL were dissolved in dichloromethane. Solution 2: 100 DL 4A was dissolved in dichloromethane at 150 mg per mL. Solution 3: 5050 DLG 2.5A was dissolved in dichloromethane at 150 mg per mL. Solution 4: 7525 DLG 4A was dissolved in dichloromethane at 150 mg per mL. Solution 5: PCL was dissolved in dichloromethane at 150 mg per mL. Solution 6: PVA was prepared at 75 mg per mL in 100 mM pH 8 phosphate buffer.

[0148] An O/W emulsion was prepared by transferring Solution 1 (0.5 mL), to a thick walled glass pressure tube. To this, lot 1 added Solution 2 (0.5 mL), lot 3 added Solution 3 (0.5 mL), lot 5 added 4 (0.5 mL), and lot 7 added Solution 5 (0.5 mL). The two solutions were then mixed by repeat pipetting. Next, Solution 6 (3.0 mL) was added, the tube was vortex mixed for 10 seconds, and was then emulsified by sonication at 30% amplitude for 1 minute with the pressure tube immersed in an ice water bath using a Branson Digital Sonifier 250. The emulsion was then added to a 50 mL beaker containing DPBS (30 mL). This was then stirred at room temperature for 2 hours to allow the dichloromethane to evaporate and for the nanocarriers to form. A portion of the nanocarriers was washed by transferring the nanocarrier suspension to a centrifuge tube and centrifuging at 75,600×g for 50 minutes, removing the supernatant, and re-suspended the pellet in DPBS. The wash procedure was repeated and then the pellet was re-suspended in DPBS to achieve a nanocarrier

suspension having a nominal concentration of 10 mg/mL on a polymer basis. The nanocarrier suspension was then filtered using a 0.22 µm PES membrane syringe filter (Millipore part number SLGP033RB), and if necessary: 0.45 µm PES membrane syringe filter (PALL part number 4614), and/or a 1.2 µm PES membrane syringe filter (PALL part number 4656). The filtered nanocarrier suspension was then stored at -20°C.

[0149] Nanocarrier size was determined by dynamic light scattering. The amount of rapamycin in the nanocarrier was determined by HPLC analysis. Filterability was determined by comparing the weight of flow through of the first sterile 0.22 µm filter to the yield to determine the actual mass of nanocarriers that passed through prior to blocking the filter, or the total through the first and only filter. The total dry-nanocarrier mass per mL of suspension was determined by a gravimetric method.

[0150] For samples 2, 4, 6 and 8, solutions were prepared as follows:

Solution 1: A polymer and rapamycin mixture was prepared by dissolving PLA-PEG-Ome at 50 mg per mL, and rapamycin at 32 mg per mL in dichloromethane. Solution 2: 100 DL 4A was dissolved in dichloromethane at 150 mg per mL. Solution 3: 5050 DLG 2.5A was dissolved in dichloromethane at 150 mg per mL. Solution 4: 7525 DLG 4A was dissolved in dichloromethane at 150 mg per mL. Solution 5: PCL was dissolved in dichloromethane at 150 mg per mL. Solution 6: Polyvinyl alcohol was prepared at 75 mg per mL in 100 mM pH 8 phosphate buffer.

[0151] An O/W emulsion was prepared by transferring Solution 1 (0.5 mL), to a thick walled glass pressure tube. To this, lot 2 added Solution 2 (0.5 mL), lot 4 added Solution 3 (0.5 mL), lot 6 added 4 (0.5 mL), and lot 8 added Solution 5 (0.5 mL). The two solutions were then mixed by repeat pipetting. The addition of PVA solution, wash, filtration and storage are the same as above.

[0152] Nanocarrier size was evaluated the same as above.

[0153] The results show a significant increase in filterability of synthetic nanocarriers comprising polyester polymers with the inclusion of SPAN 40 in the synthetic nanocarriers.

Lot number	Core polymer	Excipient	Size (nm)	Filter throughput (g NP/m ²)	Rapa load (%)	NP yield (%)
1	100 DL4A	SPAN 40	160	>148	12.65	75
2	100 DL 4A	None	197	17	10.88	71
3	5050 DLG 2.5A	SPAN 40	153	>139	13.09	70
4	5050 DLG 2.5A	None	188	59	13.40	64
5	7525 DLG 4A	SPAN 40	164	>158	11.81	78
6	7525 DLG 4A	None	196	28	11.64	73
7	Polycaprolactone	SPAN 40	164	112	10.62	75
8	Polycaprolactone	None	173	52	10.29	78

Example 13- Synthetic Nanocarriers with Low HLB Surfactant and Significant RAPA Load Results in Durable Antigen-Specific Tolerance

Materials and Methods

[0154] PLA with an inherent viscosity of 0.41 dL/g was purchased from Lakeshore Biomaterials (756 Tom

Martin Drive, Birmingham, AL 35211), product code 100 DL 4A. PLA-PEG-OMe block co-polymer with a methyl ether terminated PEG block of approximately 5,000 Da and an overall inherent viscosity of 0.50 DL/g was purchased from Lakeshore Biomaterials (756 Tom Martin Drive, Birmingham, AL 35211), product code 100 DL mPEG 5000 5CE. Rapamycin was purchased from Concord Biotech Limited (1482-1486 Trasad Road, Dholka 382225, Ahmedabad India), product code SIROLIMUS. Sorbitan monopalmitate was purchased from Sigma-Aldrich (3050 Spruce St., St. Louis, MO 63103), product code 388920. EMPROVE® Polyvinyl Alcohol (PVA) 4-88, USP (85-89% hydrolyzed, viscosity of 3.4-4.6 mPa·s) was purchased from EMD Chemicals Inc. (480 South Democrat Road Gibbstown, NJ 08027), product code 1.41350. Dulbecco's phosphate buffered saline 1X (DPBS) was purchased from Lonza (Muenchensteinerstrasse 38, CH-4002 Basel, Switzerland), product code 17-512Q.

[0155] Solutions were prepared as follows:

Solution 1: A polymer, rapamycin, and sorbitan monopalmitate mixture was prepared by dissolving PLA at 37.5 mg/mL, PLA-PEG-Ome at 12.5 mg/mL, rapamycin at 8 mg/mL, and sorbitan monopalmitate at 2.5 in dichloromethane. **Solution 2:** Polyvinyl alcohol was prepared at 50 mg/mL in 100 mM pH 8 phosphate buffer.

[0156] An O/W emulsion was prepared by combining Solution 1 (1.0 mL) and Solution 2 (3 mL) in a small glass pressure tube, vortex mixed for 10 seconds. The formulation was then homogenized by sonication at 30% amplitude for 1 minute. The emulsion was then added to an open beaker containing DPBS (30 mL). A second O/W emulsion was prepared using the same materials and method as above and then added to the same beaker containing the first emulsion and DPBS. The combined emulsion was then stirred at room temperature for 2 hours to allow the dichloromethane to evaporate and for the nanocarriers to form. A portion of the nanocarriers was washed by transferring the nanocarrier suspension to a centrifuge tube and centrifuging at 75,600×g and 4 °C for 50 minutes, removing the supernatant, and re-suspending the pellet in DPBS containing 0.25% w/v PVA. The wash procedure was repeated and then the pellet was re-suspended in DPBS containing 0.25% w/v PVA to achieve a nanocarrier suspension having a nominal concentration of 10 mg/mL on a polymer basis. The nanocarrier suspension was then filtered using a 0.22 µm PES membrane syringe filter (Millipore part number SLGP033RB). The filtered nanocarrier suspension was then stored at -20°C.

[0157] Nanocarrier size was determined by dynamic light scattering. The amount of rapamycin in the nanocarrier was determined by HPLC analysis. The total dry-nanocarrier mass per mL of suspension was determined by a gravimetric method.

Effective Diameter (nm)	Rapamycin Content (% w/w)	Nanocarrier Cone (mg/mL)
150	11.5	11.1

[0158] The ability of the synthetic nanocarriers, versus free rapamycin, to induce durable immune tolerance toward the model antigen KLH was evaluated. Groups of naive C57BL/6 mice (n=10 per group) were dosed intravenously on days 0, 7, and 14 with PBS (group 1), 50µg (~2mg/kg) free rapamycin alone or admixed with KLH (groups 2 and 3, respectively), or 50µg rapamycin encapsulated in synthetic nanocarriers alone (group 6) or admixed with KLH (groups 7 and 8) (**Fig. 8**). To determine the effects of chronic rapamycin administration group 4 received free rapamycin alone five times per week (50µg/day) from Day 0 to Day 20 or in combination with KLH administered once per week (group 5). All groups were subsequently challenged with 200 µg KLH on Days 21, 28 and 35. Sera were collected, and the anti-KLH antibody responses were measured on day 35 and 42 (after 2 and 3 injections, respectively). Efficacy was evaluated as the EC₅₀ for anti-KLH antibody titer as determined by ELISA. **Fig. 9** illustrates the protocol.

[0159] Control PBS-treated mice developed high levels of anti-KLH antibodies on days 35 and 42, after 2

and 3 challenge injections of KLH, respectively. Mice treated with free rapamycin (either weekly or daily) in the absence of KLH developed similar levels of anti-KLH antibodies as the PBS-treated group. Mice treated with synthetic nanocarriers alone or with daily free rapamycin and KLH showed a delayed response compared to the PBS control group, but the titers boosted with each challenge with KLH. These results indicate that treatment with synthetic nanocarriers alone does not induce chronic immunosuppression, and that KLH administered with daily free rapamycin, even at 5 times the total weekly dose of rapamycin as administered in synthetic nanocarriers, does not induce durable immunological tolerance.

[0160] By contrast, mice treated with synthetic nanocarriers+KLH (groups 7 and 8), that comprised a significant amount of rapamycin, developed little or no detectable anti-KLH antibodies, even after receiving three weekly post-treatment KLH challenges (for a total of 6 KLH injections), indicating durable immune tolerance. Both lots of synthetic nanocarriers were similarly effective. All groups except for those treated with synthetic nanocarrier+KLH developed anaphylactic reactions by day 42. These results indicate that tolerization to KLH induced by treatment with synthetic nanocarrier comprising a significant amount of rapamycin and KLH prevented the development of hypersensitivity reactions.

[0161] To evaluate the antigen specificity of the tolerance to KLH, all animals were challenged with OVA+CpG s.c. (35µg+20µg) in the hind limb on Days 49 and 56. **Fig. 10** shows that all animals developed similar levels of titers against OVA demonstrating that concomitant administration of the antigen with synthetic nanocarrier can yield immunological tolerance and that synthetic nanocarrier treatment does not induce chronic immunosuppression. These results demonstrate that nanocarrier-encapsulated, rather than free rapamycin, (when present at a significant amount, induced durable and antigen-specific immune tolerance when concomitantly administered with a target antigen.

Example 14- SPAN 40 Increases Filterability of Rapamycin

Materials and Methods

[0162] PLA with an inherent viscosity of 0.41 dL/g was purchased from Evonik Industries AG (Rellinghauser Straße 1-11, Essen Germany), product code 100 DL 4A. PLA-PEG-OMe block co-polymer with a methyl ether terminated PEG block of approximately 5,000 Da and an overall inherent viscosity of 0.50 DL/g was purchased from Evonik Industries AG (Rellinghauser Straße 1-11, Essen Germany), product code 100 DL mPEG 5000 5CE. Rapamycin was purchased from Concord Biotech Limited (1482-1486 Trasad Road, Dholka 382225, Ahmedabad India), product code SIROLIMUS. EMPROVE® Polyvinyl Alcohol 4-88, USP (85-89% hydrolyzed, viscosity of 3.4-4.6 mPa·s) was purchased from EMD Chemicals Inc. (480 South Democrat Road Gibbstown, NJ 08027), product code 1.41350. Dulbecco's phosphate buffered saline 1X (DPBS) was purchased from Lonza (Muenchensteinerstrasse 38, CH-4002 Basel, Switzerland), product code 17-512Q. Sorbitan monopalmitate was purchased from Croda International (300-A Columbus Circle, Edison, NJ 08837), product code SPAN 40.

[0163] Solutions were prepared as follows. **Solution 1:** A polymer and rapamycin mixture was prepared by dissolving PLA at 150 mg/mL and PLA-PEG-Ome at 50 mg/mL. **Solution 2:** A rapamycin solution was prepared at 100 mg/mL in dichloromethane. **Solution 6:** A sorbitan monopalmitate solution was prepared by dissolving SPAN 40 at 50 mg/mL in dichloromethane. **Solution 7:** Polyvinyl alcohol was prepared at 75 mg/mL in 100 mM pH 8 phosphate buffer.

[0164] O/W emulsions were prepared by adding Solution 1 (0.5mL), to a thick walled pressure tube. For lot 1, this was combined with Solution 6 (0.1 mL), and dichloromethane (0.28 mL). Lot 1 was then combined

these with Solution 2 (0.12 mL). In a similar manner, lot 2 was combined with dichloromethane (0.38 mL), and then lot 2 was combined with Solution 2 (0.12 mL). For each individual lot the total volume of the organic phase was therefore 1 mL. The combined organic phase solutions were mixed by repeat pipetting. Next, Solution 7 (3.0 mL) was added, the pressure tube was vortex mixed for 10 seconds, and was then sonicated at 30% amplitude for 1 minute with the pressure tube immersed in an ice water bath using a Branson Digital Sonifier 250. The emulsion was then added to a 50 mL beaker containing DPBS (30 mL). This was then stirred at room temperature for 2 hours to allow the dichloromethane to evaporate rapidly for the nanocarriers to form. A portion of the nanocarriers was washed by transferring the nanocarrier suspension to a centrifuge tube and centrifuging at 75,600×g and 4 °C for 50 minutes, removing the supernatant, and re-suspended the pellet in DPBS containing 0.25% w/v PVA. The wash procedure was repeated and then the pellet was re-suspended in DPBS containing 0.25% w/v PVA to achieve a nanocarrier suspension having a nominal concentration of 10 mg/mL on a polymer basis. The nanocarrier suspension was then filtered using a 0.22 µm PES membrane syringe filter (Millipore part number SLGP033RB). The filtered nanocarrier suspension was then stored at - 20°C.

[0165] The results show that the incorporation of SPAN 40 in synthetic nanocarriers increased the filterability of rapamycin.

Lot number	Rapamycin	Low HLB Surfactant	Calculated Filter Throughput (g NP/m ²)	Size (nm)	Yield (%)	Rapamycin load (%)
1	Rapamycin	SPAN 40	>117	163	60	10.41
2	Rapamycin	None	21	189	58	11.38

Example 15- Shows the Effects of the Amounts of the Components on Rapamycin Load and Synthetic Nanocarrier Filterability

Materials and Methods

[0166] PLA-PEG-OMe block co-polymer with a methyl ether terminated PEG block of approximately 5,000 Da and an overall inherent viscosity of 0.50 DL/g was purchased from Evonik Industries (Rellinghauser Straße 1-11 45128 Essen, Germany), product code 100 DL mPEG 5000 5CE. PLA with an inherent viscosity of 0.41 dL/g was purchased from Evonik Industries (Rellinghauser Straße 1-11 45128 Essen Germany), product code 100 DL 4A. Rapamycin was purchased from Concord Biotech Limited, 1482-1486 Trasad Road, Dholka 382225, Ahmedabad India. Product code SIROLIMUS. Sorbitan monopalmitate was purchased from Croda (315 Cherry Lane New Castle Delaware 19720), product code SPAN 40. Dichloromethane was purchased from Spectrum (14422 S San Pedro Gardena CA, 90248-2027). Part number M1266. EMPROVE® Polyvinyl Alcohol 4-88, (PVA), USP (85-89% hydrolyzed, viscosity of 3.4-4.6 mPa·s) was purchased from EMD Chemicals Inc. (480 South Democrat Road Gibbstown, NJ 08027), product code 1.41350. Dulbecco's Phosphate Buffered Saline (DPBS), 1X, 0.0095 M (PO₄), without calcium and magnesium, was purchased from BioWhittaker (8316 West Route 24 Mapleton, IL 61547), part number #12001, product code Lonza DPBS. Emulsification was carried out using a Branson Digital Sonifier 250 with a 1/8" tapered tip titanium probe.

[0167] Solutions were prepared as follows:

Polymer Solution: A polymer mixture was prepared by dissolving PLA-PEG-OMe (100 DL mPEG 5000 5CE) and PLA (100 DL 4A) at the indicated mg per mL in dichloromethane at a 1:3 ratio of PLA-PEG to PLA.

Rapamycin Solution: Rapamycin was dissolved at the indicated mg per 1 mL in dichloromethane. **SPAN 40 Solution:** Sorbitan monopalmitate (SPAN 40) was dissolved at the indicated mg per mL in dichloromethane. **CH₂Cl₂ Solution:** Dichloromethane (CH₂Cl₂), was sterile filtered using a 0.2µm PTFE membrane syringe filter (VWR part number 28145-491). **PVA Solution:** A polyvinyl alcohol solution was prepared by dissolving polyvinyl alcohol (EMPROVE® Polyvinyl Alcohol 4-88) at the indicated mg per 1 mL in 100 mM pH 8 phosphate buffer. **DPBS PVA Solution:** A polyvinyl alcohol and Dulbecco's phosphate buffered saline, 1X, 0.0095 M (PO₄) mixture was prepared by dissolving polyvinyl alcohol (EMPROVE® Polyvinyl Alcohol 4-88) at 2.5 mg per 1 mL in Dulbecco's phosphate buffered saline, 1X, 0.0095 M (PO₄) (Lonza DPBS).

[0168] An O/W emulsion was prepared by combining the Polymer Solution, Rapamycin Solution, SPAN 40 Solution and/or CH₂Cl₂ Solution (Total volume 1-2 mL) in a thick walled glass pressure tube. The solution was mixed by repeat pipetting. Next, PVA Solution (3 to 6 mL) was added (either as a single emulsion with 1 mL organic phase and 3 mL aqueous PVA Solution, or as two single emulsions prepared one after the other). The formulation was vortex mixed for ten seconds, and then sonicated with the pressure tube immersed in an ice bath for 1 minute at 30% amplitude. The emulsion was then added to an open 50 mL beaker containing Lonza DPBS (30 mL). This was then stirred at room temperature for 2 hours to allow the dichloromethane to evaporate and for the nanocarriers to form. A portion of the nanocarriers were washed by transferring the nanocarrier suspension to a centrifuge tube and centrifuging at 75,600×g and 4 °C for 50 minutes, removing the supernatant, and re-suspending the pellet in DPBS PVA Solution. The wash procedure was repeated and then the pellet was re-suspended in DPBS PVA Solution to achieve a nanocarrier suspension having a nominal concentration of 10 mg per mL on a polymer basis. The nanocarrier formulation was filtered using a 0.22µm PES membrane syringe filter (Millex part number SLGP033RS). The mass of the nanocarrier solution filter throughput was measured. The filtered nanocarrier solution was then stored at -20°C.

[0169] Filterability is given as g/m² of filter membrane surface area, of measured nanocarrier passing through one 33 mm PES membrane 0.22 µm syringe filter from Millipore, part number SLGP033RB.

[0170] The results show the amount of various components in a number of synthetic nanocarriers that can result in initial sterile filterable synthetic nanocarriers with an amount of rapamycin that is expected to be efficacious *in vivo*.

Lot#	Polymer (mg per mL)	SPAN 40 (mg per mL)	Rapamycin (mg per mL)	PVA (mg per mL)	Size (nm)	Filterability (g NP/m ²)	Yield	wt% HLB/Rapa	wt% HLB/Polymer
1 ^a	50	0	8	62.5	135	52	70.7	0.00	0.00
2 ^a	50	0.1	8	62.5	135	26	68.6	1.23	0.20
3 ^a	50	0.25	8	62.5	148	27	70.9	3.03	0.50
4 ^a	50	0.5	8	62.5	166	146	73.2	5.88	0.99
5 ^a	50	1	8	62.5	147	151	75.7	11.11	1.96
6 ^a	50	1.5	8	62.5	161	146	72.2	15.79	2.91
7 ^a	50	2.5	8	62.5	149	176	85.0	23.81	4.76
8 ^a	50	2.5	8	50	182	209	103.5	23.81	4.76
9 ^a	50	2.5	8	75	132	155	76.7	23.81	4.76
10 ^a	50	3	8	62.5	143	140	69.4	27.27	5.66

Lot#	Polymer (mg per mL)	SPAN 40 (mg per mL)	Rapamycin (mg per mL)	PVA (mg per mL)	Size (nm)	Filterability (g NP/m ²)	Yield	wt% HLB/Rapa	wt% HLB/Polymer
11 ^a	62.5	3	8	62.5	151	205	80.9	27.27	4.58
12 ^a	37.5	3	8	62.5	139	203	60.9	27.27	7.41
13 ^a	50	4.5	8	62.5	149	149	73.6	36.00	8.26
14 ^a	50	5	6.66	50	148	193	94.4	42.88	9.09
15 ^a	50	5	8.33	50	176	176	86.2	37.48	9.09
16 ^a	50	10	8	50	173	38	66.1	55.56	16.67
17	100	10	11.32	75	153	178	88.2	46.90	909
18	100	10	14.16	75	160	200	98.9	41.39	909
19	100	10	17	75	177	182	101.0	3704	909
20	100	7.5	24	75	188	125	70.4	23.81	6.98
21	75	11.25	30	75	197	17	82.5	27.27	13.04
22	100	15	32	75	201	17	108.1	31.91	13.04
23	100	15	40	75	217	9	82.6	27.27	13.04
24	100	15	40	75	193	14	116.5	27.27	13.04
^a These formulations were prepared with 2 mL organic phase, 6 mL PVA Solution.									

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Patentkrav

1. Sammensætning, som omfatter:

syntetiske nanobærere, der omfatter et hydrofobt polyesterbæremateriale og rapamycin;
hvor rapamycin er til stede i de syntetiske nanobærere i en stabil, supermættet mængde; og
eventuelt hvor de syntetiske nanobærere er indledningsvis sterilfiltrerbare.

2. Sammensætning ifølge krav 1, hvor:

i) rapamycin er til stede i en stabil, supermættet mængde, der er mindre end 50 vægt-%;

ii) rapamycin er til stede i en stabil, supermættet mængde, der er mindre end 45 vægt-%;

iii) rapamycin er til stede i en stabil, supermættet mængde, der er mindre end 40 vægt-%;

iv) rapamycin er til stede i en stabil, supermættet mængde, der er mindre end 35 vægt-%;

v) rapamycin er til stede i en stabil, supermættet mængde, der er mindre end 30 vægt-%;

vi) rapamycin er til stede i en stabil, supermættet mængde, der er mindre end 25 vægt-%;

vii) rapamycin er til stede i en stabil, supermættet mængde, der er mindre end 20 vægt-%;

viii) rapamycin er til stede i en stabil, supermættet mængde, der er mindre end 15 vægt-%;

ix) rapamycin er til stede i en stabil, supermættet mængde, der er mindre end 10 vægt-%; eller

x) rapamycin er til stede i en stabil, supermættet mængde, der er mere end 7 vægt-%.

3. Sammensætning ifølge et hvilket som helst af ovennævnte krav, hvor mængden af det hydrofobe polyesterbæremateriale i de syntetiske nanobærere er 5-95 vægt-% hydrofobt polyesterbæremateriale/samlede faststoffer, fortrinsvis hvor mængden af hydrofobt polyesterbæremateriale i de syntetiske nanobærere er 60-95 vægt-% hydrofobt

polyesterbæremateriale/samlede faststoffer.

4. Sammensætning ifølge et hvilket som helst af ovennævnte krav, hvor sammensætningen er indledningsvis sterilfiltrerbar
5 gennem et 0,22 µm-filter.
5. Sammensætning ifølge et hvilket som helst af ovennævnte krav, hvor middelværdien af en partikelstørrelsesfordeling opnået ved anvendelse af dynamisk lysspredning af de syntetiske
10 nanobærere er en diameter større end 120 nm, fortrinsvis hvor diameteren er større end 150 nm.
6. Sammensætning ifølge krav 5, hvor diameteren er større end 200 nm.
15
7. Sammensætning ifølge krav 6, hvor diameteren er større end 250 nm.
8. Sammensætning ifølge et hvilket som helst af kravene 5-7,
20 hvor diameteren er mindre end 300 nm.
9. Sammensætning ifølge et hvilket som helst af kravene 5-6, hvor diameteren er mindre end 250 nm.
- 25 10. Sammensætning ifølge krav 5, hvor diameteren er mindre end 200 nm.
11. Sammensætning ifølge et hvilket som helst af ovennævnte krav, hvor rapamycin er indkapslet i de syntetiske nanobærere.
30
12. Sammensætning ifølge et hvilket som helst af ovennævnte krav, hvor:
i) sammensætningen yderligere omfatter et antigen; eller
ii) sammensætningen yderligere omfatter et antigen, og hvor
35 antigenet er blandet med de syntetiske nanobærere i sammensætningen.
13. Sammensætning ifølge et hvilket som helst af ovennævnte

krav, hvor sammensætningen yderligere omfatter en farmaceutisk acceptabel bærer.

14. Sammensætning ifølge et hvilket som helst af ovennævnte krav, hvor de syntetiske nanobærere yderligere omfatter et ikke-ionisk overfladeaktivt stof med en HLB-værdi mindre end eller lig med 10.

15. Sammensætning ifølge krav 14, hvor det ikke-ioniske overfladeaktive stof med en HLB-værdi mindre end eller lig med 10 er indkapslet i de syntetiske nanobærere, er til stede på overfladen af de syntetiske nanobærere eller begge.

16. Fremgangsmåde til fremstilling af syntetiske nanobærere, der omfatter et hydrofobt polyesterbæremateriale og rapamycin, som omfatter:

opnåelse eller tilvejebringelse af det hydrofobe polyesterbæremateriale,
opnåelse eller tilvejebringelse af rapamycin i en mængde, der overstiger mætningsgrænsen for rapamycin,
kombinering af det hydrofobe polyesterbæremateriale og rapamycin og
stabilisering af rapamycin.

17. Fremgangsmåde ifølge krav 16, hvor rapamycin stabiliseres ved tilsætning af et ikke-ionisk overfladeaktivt stof med HLB-værdier, der er mindre end eller lig med 10.

18. Fremgangsmåde ifølge krav 17 eller sammensætning ifølge krav 14 eller 15, hvor:

- i) det ikke-ioniske overfladeaktive stof med en HLB-værdi mindre end eller lig med 10 omfatter en sorbitanester, fedtalkohol, fedtsyreester, ethoxyleret fedtalkohol, poloxamer eller en fedtsyre;
- ii) det ikke-ioniske overfladeaktive stof med en HLB-værdi mindre end eller lig med 10 omfatter en sorbitanester, fedtalkohol, fedtsyreester, ethoxyleret fedtalkohol, poloxamer eller en fedtsyre, og hvor det ikke-ioniske overfladeaktive stof

med en HLB-værdi mindre end eller lig med 10 omfatter SPAN 40, SPAN 20, oleylalkohol, stearylalkohol, isopropylpalmitat, glycerolmonostearat, BRIJ 52, BRIJ 93, pluronisk P-123, pluronisk L-31, palmitinsyre, dodecansyre, glyceryltripalmitat

5 eller glyceryltrilinoleat; eller

iii) det ikke-ioniske overfladeaktive stof med en HLB-værdi mindre end eller lig med 10 er SPAN 40.

19. Fremgangsmåde ifølge et hvilket som helst af kravene 16-18
10 eller sammensætning ifølge et hvilket som helst af kravene 14, 15 eller 18, hvor:

i) mængden af ikke-ionisk overfladeaktivt stof med en HLB-værdi mindre end eller lig med 10 er $\geq 0,1$ men ≤ 15 vægt-% ikke-ionisk overfladeaktivt stof med en HLB-værdi mindre end eller lig med
15 10/vægt hydrofobt polyesterbæremateriale;

ii) mængden af ikke-ionisk overfladeaktivt stof med en HLB-værdi mindre end eller lig med 10 er ≥ 1 men ≤ 13 vægt-% ikke-ionisk overfladeaktivt stof med en HLB-værdi mindre end eller lig med 10/vægt hydrofobt polyesterbæremateriale; eller

20 iii) mængden af ikke-ionisk overfladeaktivt stof med en HLB-værdi mindre end eller lig med 10 er ≥ 1 men ≤ 9 vægt-% ikke-ionisk overfladeaktivt stof med en HLB-værdi mindre end eller lig med 10/vægt hydrofobt polyesterbæremateriale.

25 20. Fremgangsmåde ifølge et hvilket som helst af kravene 16-19 eller sammensætning ifølge et hvilket som helst af kravene 1-15, 18 eller 19, hvor:

i) det hydrofobe polyesterbæremateriale omfatter PLA, PLG, PLGA eller polycaprolacton; eller

30 ii) det hydrofobe polyesterbæremateriale omfatter PLA, PLG, PLGA eller polycaprolacton, og hvor det hydrofobe polyesterbæremateriale yderligere omfatter PLA-PEG, PLGA-PEG eller PCL-PEG.

35 21. Fremgangsmåde ifølge krav 16, hvor rapamycin stabiliseres med hurtig opløsningsmiddelfordampning af det kombinerede hydrofobe polyesterbæremateriale og rapamycin ved tilstedeværelse af et opløsningsmiddel.

22. Fremgangsmåde ifølge et hvilket som helst af kravene 16-21, hvor fremgangsmåden yderligere omfatter bestemmelse af mætningsgrænsen for rapamycin i det hydrofobe polyesterbæremateriale.

23. Fremgangsmåde ifølge krav 20, hvor fremgangsmåden yderligere omfatter bestemmelse af mætningsgrænsen for rapamycin i det hydrofobe polyesterbæremateriale ved anvendelse af formlen:

$$RAPA-indhold = V(0,008C_{PVA} + 0,072C_{pol}) \text{ eller } RAPA-indhold = V(0,008C_{PVA} + 0,084C_{pol});$$

hvor C_{PVA} er massekoncentrationen af PVA, C_{pol} er den kombinerede massekoncentration af polymererne, og V er volumenet af nanobærersuspensionen efter afsluttet fordampning.

24. Fremgangsmåde ifølge et hvilket som helst af kravene 16-23, hvor:

- i) mængden af rapamycin overstiger mætningsgrænsen med mere end 1 %;
- ii) mængden af rapamycin overstiger mætningsgrænsen med mindst 5 %;
- iii) mængden af rapamycin overstiger mætningsgrænsen med mindst 10 %;
- iv) mængden af rapamycin overstiger mætningsgrænsen med mindst 15 %;
- v) mængden af rapamycin overstiger mætningsgrænsen med mindst 20 %;
- vi) mængden af rapamycin overstiger mætningsgrænsen med mindst 25 %;
- vii) mængden af rapamycin overstiger mætningsgrænsen med mindst 30 %; eller
- viii) mængden af rapamycin overstiger mætningsgrænsen med mindst 35 %.

25. Fremgangsmåde ifølge et hvilket som helst af kravene 16-24, hvor:

- i) fremgangsmåden yderligere omfatter filtrering af den

resulterende sammensætning; eller

ii) fremgangsmåden yderligere omfatter filtrering af den resulterende sammensætning, og hvor filtreringen omfatter filtrering gennem et 0,22 µm-filter.

5

26. Sammensætning frembragt ved hjælp af fremgangsmåden ifølge et hvilket som helst af kravene 16-25.

27. Kit, der omfatter:

10 sammensætningen ifølge et hvilket som helst af ovennævnte krav.

28. Kit ifølge krav 27, hvor kittet, når sammensætningen ikke omfatter antigen, yderligere omfatter et antigen.

15 29. Kit ifølge krav 28, hvor sammensætningen og antigenet er indeholdt i separate beholdere, eller hvor sammensætningen og antigenet er indeholdt i den samme beholder.

20 30. Kit ifølge et hvilket som helst af kravene 27-29, som yderligere omfatter en brugsanvisning.

DRAWINGS

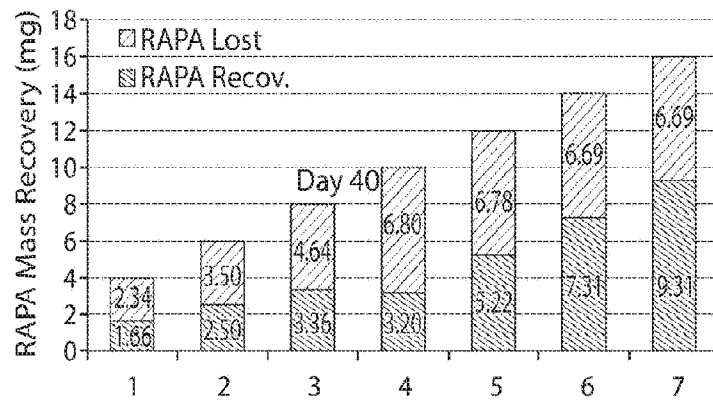


Fig. 1

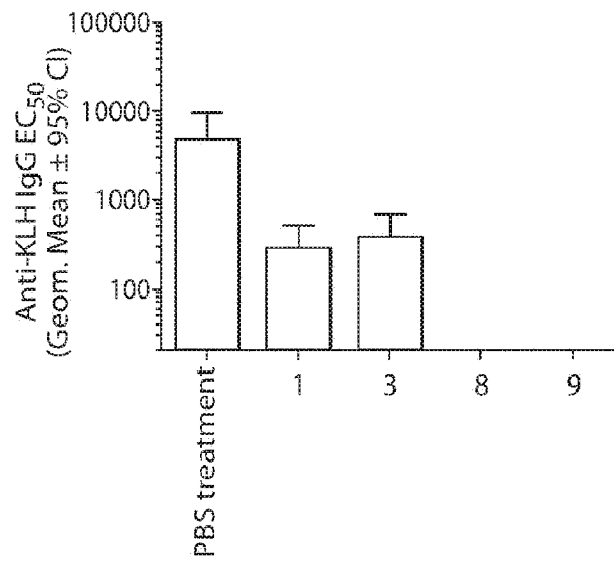


Fig. 2

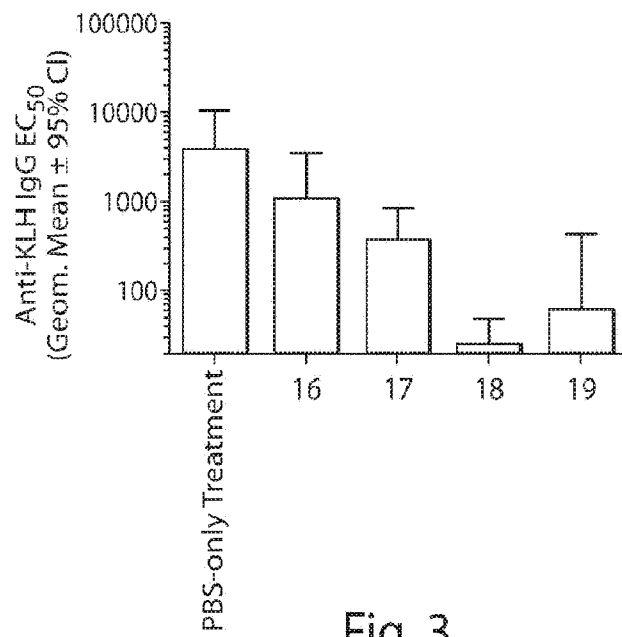


Fig. 3

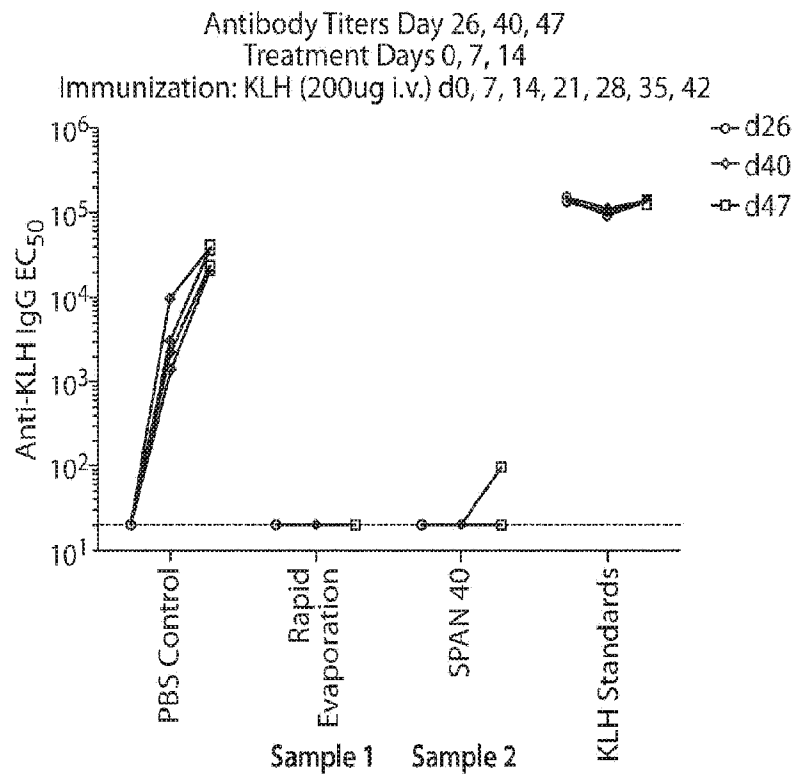


Fig. 4

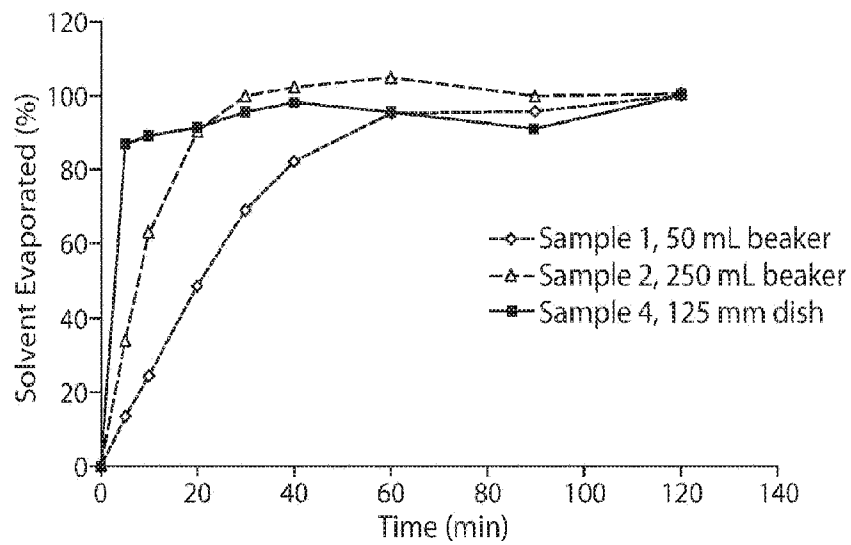


Fig. 5

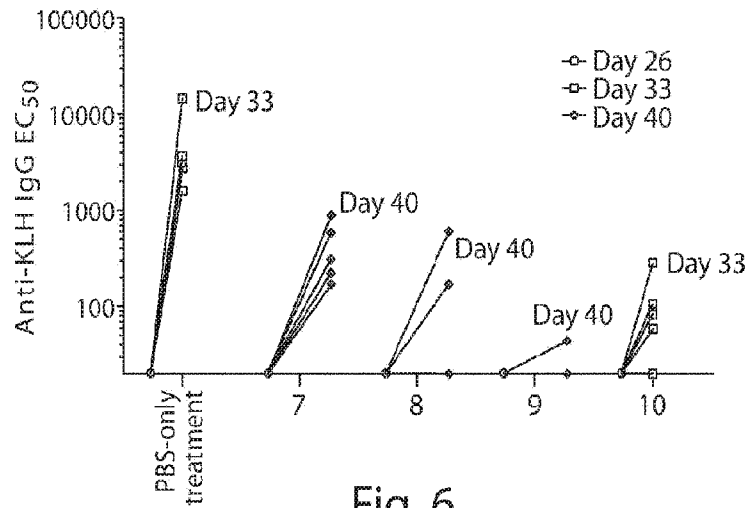


Fig. 6

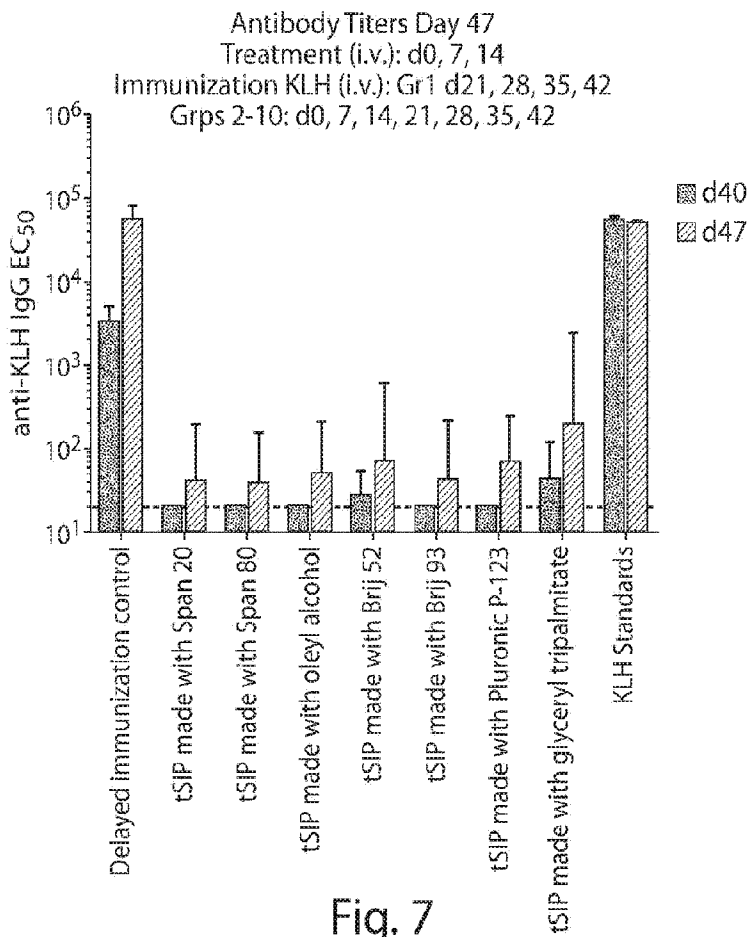


Fig. 7

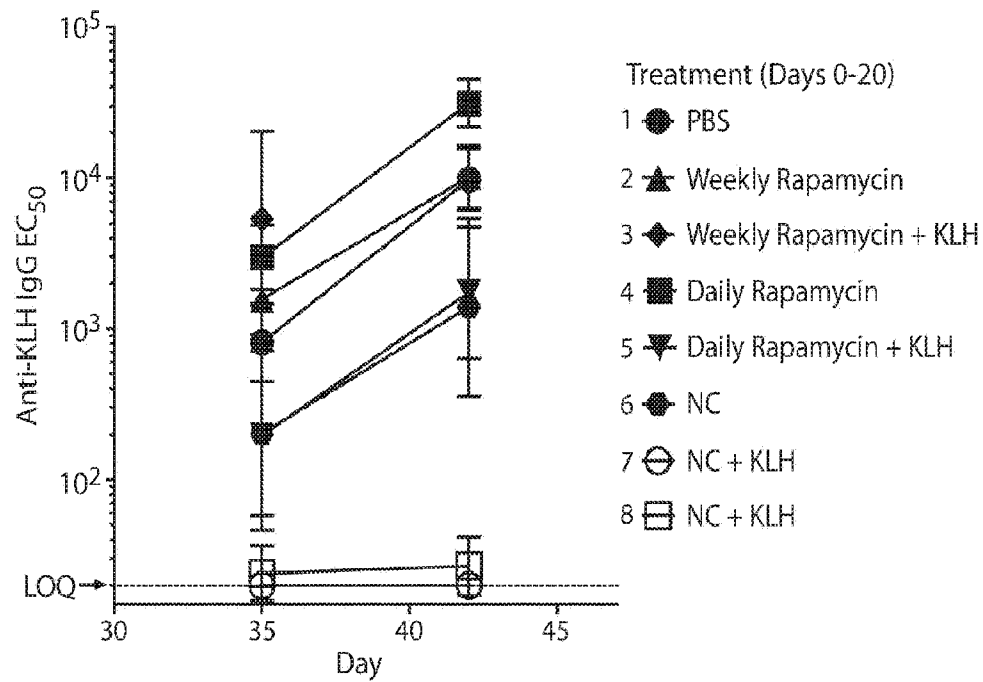


Fig. 8

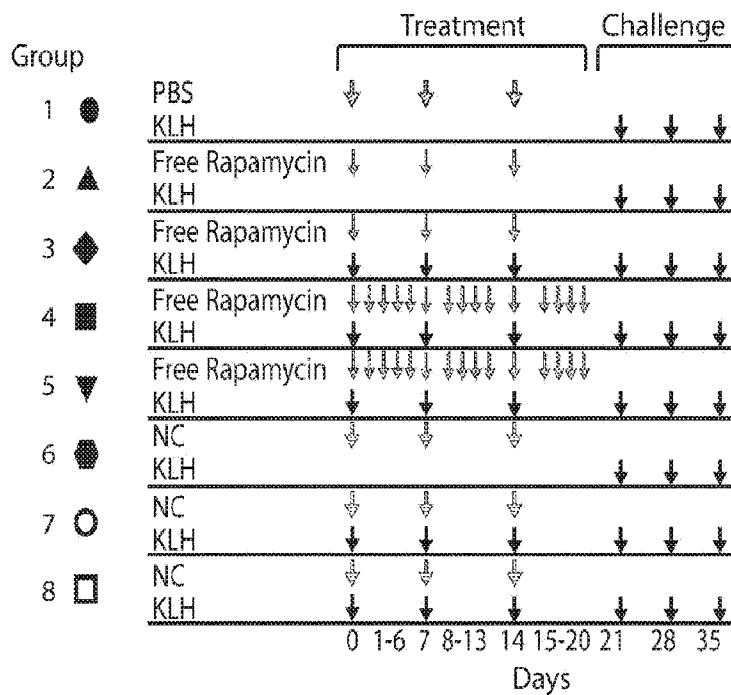


Fig. 9

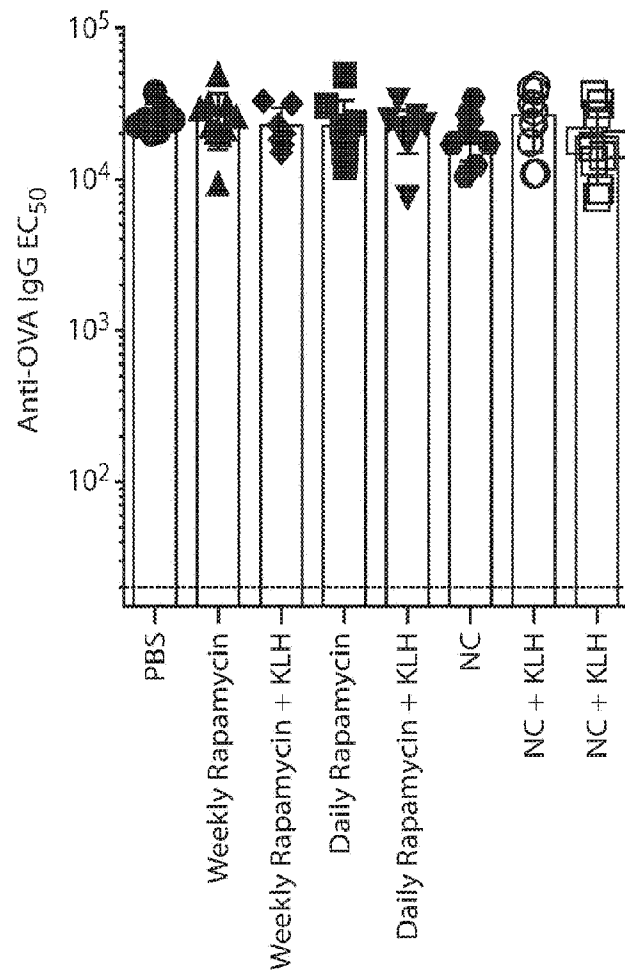


Fig. 10