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Canadian Intellectual Property Office

CA 3013880 A1 2017/08/17

(21) 3 013 880

(12) DEMANDE DE BREVET CANADIEN
CANADIAN PATENT APPLICATION

(13) A1

(86) Date de dépôt PCT/PCT Filing Date: 2017/02/10
(87) Date publication PCT/PCT Publication Date: 2017/08/17
(85) Entrée phase nationale/National Entry: 2018/08/07
(86) N° demande PCT/PCT Application No.: IB 2017/050762
(87) N° publication PCT/PCT Publication No.: 2017/137953
(30) Priorité/Priority: 2016/02/10 (US62/293,617)

(51) Cl.Int./Int.Cl. *A61K 47/54* (2017.01), *A61K 31/337* (2006.01), *A61K 39/00* (2006.01), *A61K 39/395* (2006.01), *A61K 47/69* (2017.01), *A61P 35/00* (2006.01), *C07K 16/28* (2006.01)

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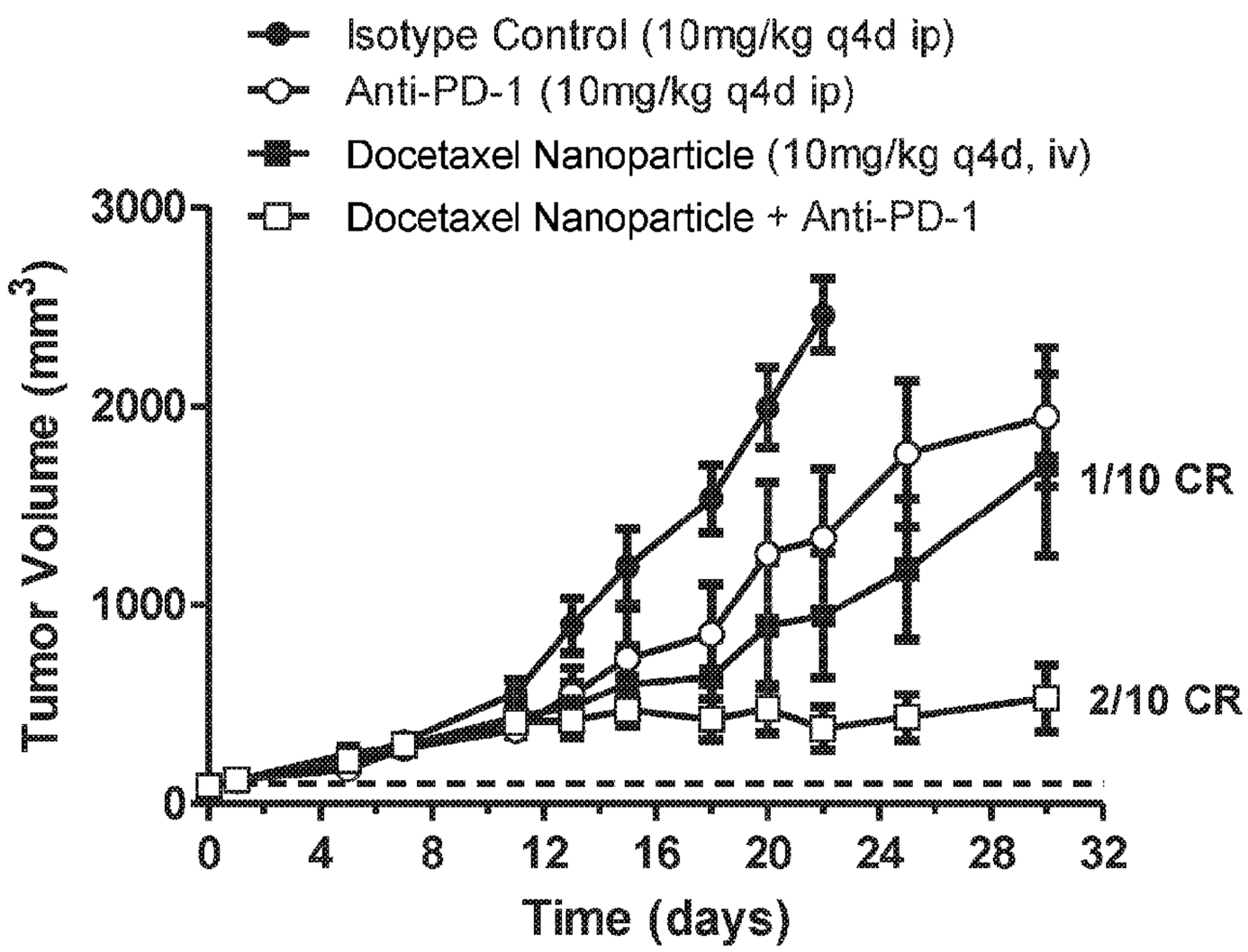
(54) Titre : NANOPARTICULES THERAPEUTIQUES COMPRENANT UN AGENT THERAPEUTIQUE, ET PROCEDES DE FABRICATION ET METHODES D'UTILISATION DE CES DERNIERES

(54) Title: THERAPEUTIC NANOPARTICLES COMPRISING A THERAPEUTIC AGENT AND METHODS OF MAKING AND USING SAME

FIG. 3



(57) Abrégé/Abstract:
The present disclosure generally relates to nanoparticles comprising an antibody, such as an anti-PD-1 antibody. Other aspects include methods of making and using such nanoparticles. In an embodiment, the nanoparticles comprise a diblock poly(lactic) acid-poly(ethylene)glycol (PLA-PEG) copolymer, a chemotherapeutic agent, and a prostate-specific membrane antigen (PSMA) targeting ligand.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
17 August 2017 (17.08.2017)

WIPO | PCT

(10) International Publication Number
WO 2017/137953 A1

(51) International Patent Classification:
A61K 47/54 (2017.01) *A61K 39/395* (2006.01)
A61K 47/69 (2017.01) *A61K 39/00* (2006.01)
A61K 31/337 (2006.01) *A61P 35/00* (2006.01)
C07K 16/28 (2006.01)

(21) International Application Number:
PCT/IB2017/050762

(22) International Filing Date:
10 February 2017 (10.02.2017)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/293,617 10 February 2016 (10.02.2016) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

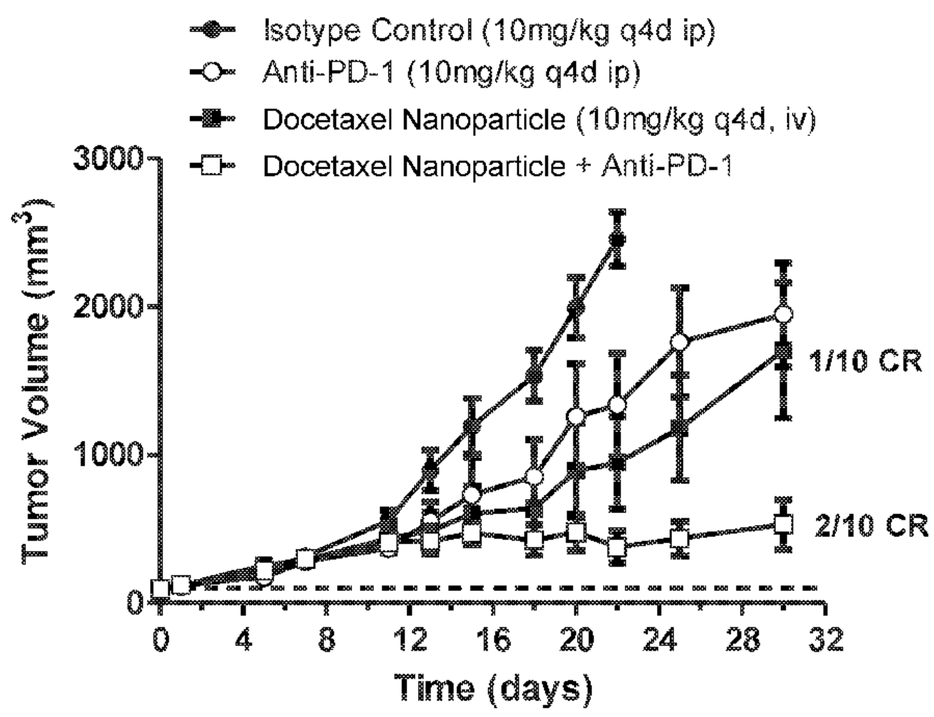
Published:

— *with international search report (Art. 21(3))*

— *with sequence listing part of description (Rule 5.2(a))*

(54) Title: THERAPEUTIC NANOPARTICLES COMPRISING A THERAPEUTIC AGENT AND METHODS OF MAKING AND USING SAME

FIG. 3



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THERAPEUTIC NANOPARTICLES COMPRISING A THERAPEUTIC AGENT AND METHODS OF MAKING AND USING SAME

BACKGROUND

Systems that deliver certain drugs to a patient (*e.g.*, targeted to a particular tissue or cell type or targeted to a specific diseased tissue but not normal tissue) or that control release of drugs have long been recognized as beneficial.

For example, therapeutics that include an active drug and that are, *e.g.*, targeted to a particular tissue or cell type or targeted to a specific diseased tissue but not to normal tissue, may reduce the amount of the drug in tissues of the body that are not targeted. This is particularly important when treating a condition such as cancer where it is desirable that a cytotoxic dose of the drug is delivered to cancer cells without killing the surrounding non-cancerous tissue. Effective drug targeting may reduce the undesirable and sometimes life threatening side effects common in anticancer therapy. In addition, such therapeutics may allow drugs to reach certain tissues they would otherwise be unable to reach.

Therapeutics that offer controlled release and/or targeted therapy also must be able to deliver an effective amount of drug, which is a known limitation in other nanoparticle delivery systems. For example, it can be a challenge to prepare nanoparticle systems that have an appropriate amount of drug associated with each nanoparticle, while keeping the size of the nanoparticles small enough to have advantageous delivery properties.

Therapeutic delivery of checkpoint inhibitors offer promising treatment of cancers. These therapeutic agents require efficient and nontoxic delivery methods. However, there are significant challenges in delivery of this class of agents, including persevering antibody integrity from degradation. Nanoparticle formulations that include such antibodies are often hindered by undesirable properties, *e.g.*, burst release profiles and degradation of the antibody.

Accordingly, a need exists for nanoparticle therapeutics and methods of making such nanoparticles that are capable of delivering antibodies, while also preserving antibody efficacy and potency.

SUMMARY OF INVENTION

Described herein are therapeutic and/or pharmaceutically acceptable polymeric nanoparticles comprising a therapeutic agent, where the therapeutic nanoparticles and an anti- PD-1 antibody are administered to a patient. In some embodiments, the nanoparticle encapsulates a therapeutic agent (*e.g.*, docetaxel), and the therapeutic nanoparticle and an anti-PD-1 antibody are administered side-by-side to a patient for treatment. In some embodiments, the anti-PD-1 antibody and a therapeutic agent (*e.g.*, docetaxel) are encapsulated within a nanoparticle. In some embodiments, the therapeutic nanoparticle and an anti-PD-1 antibody are administered (side-by-side, or with the anti-PD-1 antibody and therapeutic agent within the nanoparticle) to a patient with squamous non small cell lung cancer. In some embodiments, the therapeutic nanoparticle also includes a hydrophobic counter ion agent.

Contemplated nanoparticles may include an antibody that acts as a checkpoint inhibitor. For example, contemplated nanoparticles may include an anti-PD-1 antibody. It should be appreciated that the nanoparticle may incorporate a hydrophobic counter ion agent. It should also be appreciated that the antibody may be encapsulated within the nanoparticle, or may be attached to the nanoparticle, or may be administered side-by-side with the therapeutic nanoparticle.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is flow chart for an emulsion process for forming a disclosed nanoparticle.

Figures 2A and 2B show flow diagrams for a disclosed emulsion process.

Figure 3: Is a Spider plot that showings mean tumor volume over time following treatment initiation with Isotype control (10mg/kg q4d ip); Anti-PD-1 (10mg/kg q4d ip); docetaxel nanoparticle (10mg/kg q4d ip); and docetaxel nanoparticle + Anti-PD-1.

Figure 4: Is a Spider plot showing tumor growth of individual mice treated with anti-PD-1.

Figure 5: Is a Spider plot showing tumor growth of individual mice treated with docetaxel nanoparticle.

Figure 6: Is a Spider plot showing tumor growth of individual mice treated with anti-PD-1 and docetaxel nanoparticle.

Figure 7: Is a Spider plot showing, in a repeat efficacy study, mean tumor volume over time following treatment initiation with Isotype control (10mg/kg q4d ip); Anti-PD-1 (10mg/kg q4d ip); docetaxel nanoparticle (10mg/kg q4d ip); and docetaxel nanoparticle + Anti-PD-1.

Figure 8: Is a Spider plot showing that there was no significant effect of treatment on body weight.

Figure 9: Is a Spider plot of data showing mean tumor volume over time following treatment initiation with Isotype control (10mg/kg iv q4d); Anti-PD-1 (10mg/kg iv q4d); docetaxel (taxotere -2.5mg/kg iv q4d); and docetaxel (taxotere) + Anti-PD-1.

Figure 10: Is a Spider plot showing that there was no significant effect of treatment on body weight.

Figure 11: Is a Spider plot showing tumor growth of individual mice treated with isotype control.

Figure 12: Is a Spider plot showing tumor growth of individual mice treated with anti-PD-1 (repeat study).

Figure 13: Is a Spider plot showing tumor growth of individual mice treated with decetaxel nanoparticle (repeat study).

Figure 14: Is a Spider plot showing tumor growth of individual mice treated with anti-PD-1 and docetaxel nanoparticle (repeat study).

Figure 15: Is a Spider plot showing tumor growth of individual mice treated with docetaxel.

Figure 16: Is a Spider plot showing tumor growth of individual mice treated with anti-PD-1 and docetaxel.

DETAILED DESCRIPTION

Described herein are polymeric nanoparticles that include at least one therapeutic agent (*e.g.,* docetaxel, or docetaxel and an anti-PD-1 antibody), and methods of making and using such therapeutic nanoparticles. In some embodiments, a disclosed nanoparticle includes an antibody, such as an anti-PD-1 antibody that is a checkpoint inhibitor. In some embodiments, the therapeutic nanoparticle encapsulates a therapeutic agent (*e.g.*, docetaxel), and the nanoparticle is administered side-by-side

(or coadmininstered) with an anti-PD-1 antibody to a patient, for example, a patient with squamous non small cell lung cancer. In some embodiments inclusion (*i.e.*, doping) of a hydrophobic acid (*e.g.*, a fatty acid and/or a bile acid) in a disclosed nanoparticle and/or included in a nanoparticle preparation process may result in nanoparticles that include improved drug loading. Furthermore, in certain embodiments, nanoparticles that include and/or are prepared in the presence of the hydrophobic acid may exhibit improved controlled release properties. For example, disclosed nanoparticles may more slowly release the antibody therapeutic agent as compared to nanoparticles prepared in the absence of the hydrophobic acid.

Contemplated herein are nanoparticles that include antibodies, such as anti-PD-1 antibodies, for example, nivolumab, pembrolizumab, ipilimumab, etc.

In still another aspect, a pharmaceutically acceptable composition is provided. The pharmaceutically acceptable composition may comprise a plurality of therapeutic nanoparticles as described herein and a pharmaceutically acceptable excipient. In some embodiments, the pharmaceutical composition comprises a nanoparticle encapsulating a therapeutic agent (*e.g.,* docetaxel) and an anti-PD-1 antibody. In some embodiments, the anti- PD-1 antibody is not encapsulated in the nanoparticle. In some embodiments, the anti-PD-1 antibody is encapsulated in the nanoparticle.

In yet another aspect, a method of treating a cancer patient in need thereof is provided. The method comprises administering to the patient a therapeutically effective amount of a composition comprising a therapeutic nanoparticle as described herein. In some embodiments, the composition comprises a therapeutic nanoparticle that encapsulates at least one therapeutic agent (*e.g.,* docetaxel, or docetaxel and an anti-PD-1 antibody). In some embodiments, the composition comprises a therapeutic nanoparticle that encapsulates at least one therapeutic agent (*e.g.,* docetaxel), and is administered side-by-side with an anti-PD-1 antibody.

Without wishing to be bound by any theory, it is believed that the disclosed nanoparticle formulations that include a hydrophobic acid (*e.g.,* fatty acid and/or bile acid) have significantly improved formulation properties (*e.g.,* drug loading and/or release profile) through formation of a hydrophobic ion-pair (HIP) or hydrophobic counter ion, between a therapeutic agent and an acid. As used herein, a HIP is a pair of oppositely charged ions held together by Coulombic attraction. In some embodiments, the antibody within the nanoparticle may be associated with a hydrophobic counter ion agent. It should be appreciated that the term “ion- agent” or

"ion-pair" is not to be limited to a 1:1 ratio, but instead refers to the ions of opposite charges, in any ratio, to be attracted to one another. For example, a therapeutic agent or antibody with eight negative charges may be "paired" with eight positively charged molecules. Or, a therapeutic agent or antibody with eight positive charges may be "paired" with eight negative charges. Thus, as used herein, an ion-pair is a pair of oppositely charged ions held together by Coulombic attraction. Ion-pair formation, as contemplated herein, can result in nanoparticles having for example, increased drug loading. Slower release of the therapeutic agent or antibody from the nanoparticles may also occur, for example in some embodiments, due to a decrease in the therapeutic agent's solubility in aqueous solution. Furthermore, complexing the therapeutic agent or antibody with large hydrophobic counter ions may slow diffusion of the therapeutic agent within the polymeric matrix. Advantageously, ion-pair formation occurs without the need for covalent conjugation of the hydrophobic group to the therapeutic agent or antibody.

Nanoparticles disclosed herein include one, two, three or more biocompatible and/or biodegradable polymers. For example, a contemplated nanoparticle may include about 35 to about 99.75 weight percent, in some embodiments about 50 to about 99.75 weight percent, in some embodiments about 50 to about 99.5 weight percent, in some embodiments about 50 to about 99 weight percent, in some embodiments about 50 to about 98 weight percent, in some embodiments about 50 to about 97 weight percent, in some embodiments about 50 to about 96 weight percent, in some embodiments about 50 to about 95 weight percent, in some embodiments about 50 to about 94 weight percent, in some embodiments about 50 to about 93 weight percent, in some embodiments about 50 to about 92 weight percent, in some embodiments about 50 to about 91 weight percent, in some embodiments about 50 to about 90 weight percent, in some embodiments about 50 to about 85 weight percent, in some embodiments about 60 to about 85 weight percent, in some embodiments about 65 to about 85 weight percent, and in some embodiments about 50 to about 80 weight percent of one or more block copolymers that include a biodegradable polymer and poly(ethylene glycol) (PEG), and about 0 to about 50 weight percent of a biodegradable homopolymer.

In some embodiments, disclosed nanoparticles may include about 0.2 to about 35 weight percent, about 0.2 to about 20 weight percent, about 0.2 to about 10 weight percent, about 0.2 to about 5 weight percent, about 0.5 to about 5 weight percent, about

0.75 to about 5 weight percent, about 1 to about 5 weight percent, about 2 to about 5 weight percent, about 3 to about 5 weight percent, about 1 to about 20 weight percent, about 2 to about 20 weight percent, about 5 to about 20 weight percent, about 1 to about 15 weight percent, about 2 to about 15 weight percent, about 3 to about 15 weight percent, about 4 to about 15 weight percent, about 5 to about 15 weight percent, about 1 to about 10 weight percent, about 2 to about 10 weight percent, about 3 to about 10 weight percent, about 4 to about 10 weight percent, about 5 to about 10 weight percent, about 10 to about 30 weight percent, or about 15 to about 25 weight percent of a therapeutic agent.

In certain embodiments, disclosed nanoparticles comprise a hydrophobic acid (*e.g.,* a fatty acid and/or bile acid) and/or are prepared by a process that includes a hydrophobic acid. Such nanoparticles may have a higher drug loading than nanoparticles prepared by a process without a hydrophobic acid. For example, drug loading (*e.g.,* by weight) of disclosed nanoparticles prepared by a process comprising the hydrophobic acid may be between about 2 times to about 10 times higher, or even more, than disclosed nanoparticles prepared by a process without the hydrophobic acid. In some embodiments, the drug loading (by weight) of disclosed nanoparticles prepared by a first process comprising the hydrophobic acid may be at least about 2 times higher, at least about 3 times higher, at least about 4 times higher, at least about 5 times higher, or at least about 10 times higher than disclosed nanoparticles prepared by a second process, where the second process is identical to the first process except that the second process does not include the hydrophobic acid.

Any suitable hydrophobic acid is contemplated. In some embodiments, the hydrophobic acid may be a carboxylic acid (*e.g.,* a monocarboxylic acid, dicarboxylic acid, tricarboxylic acid, or the like), a sulfinic acid, a sulfenic acid, or a sulfonic acid. In some cases, a contemplated hydrophobic acid may include a mixture of two or more acids. In some cases, a salt of a hydrophobic acid may be used in a formulation.

For example, a disclosed carboxylic acid may be an aliphatic carboxylic acid (*e.g.,* a carboxylic acid having a cyclic or acyclic, branched or unbranched, hydrocarbon chain). Disclosed carboxylic acids may, in some embodiments, be substituted with one or more functional groups including, but not limited to, halogen (*i.e.,* F, Cl, Br, and I), sulfonyl, nitro, and oxo. In certain embodiments, a disclosed carboxylic acid may be unsubstituted.

Exemplary carboxylic acids may include a substituted or unsubstituted fatty acid (*e.g.,* C_6-C_{50} fatty acid). In some instances, the fatty acid may be a C_{10}-C_{20} fatty acid. In other instances, the fatty acid may be a C_{15}-C_{20} fatty acid. The fatty acid may, in some cases, be saturated. In other embodiments, the fatty acid may be unsaturated. For instance, the fatty acid may be a monounsaturated fatty acid or a polyunsaturated fatty acid. In some embodiments, a double bond of an unsaturated fatty acid group can be in the cis conformation. In some embodiments, a double bond of an unsaturated fatty acid can be in the *trans* conformation. Unsaturated fatty acids include, but are not limited to, omega-3, omega-6, and omega-9 fatty acids.

Non-limiting examples of saturated fatty acids include caproic acid, enanthic acid, caprylic acid, pelargonic acid, capric acid, undecanoic acid, lauric acid, tridecanoic acid, myristic acid, pentadecanoic acid, palmitic acid, margaric acid, stearic acid, nonadecanoic acid, arachidic acid, heneicosanoic acid, behenic acid, tricosanoic acid, lignoceric acid, pentacosanoic acid, cerotic acid, heptacosanoic acid, montanic acid, nonacosanoic acid, melissic acid, henatriacontanoic acid, lacceroic acid, psyllic acid, geddic acid, ceroplastic acid, hexatriacontanoic acid, and combinations thereof.

Non-limiting examples of unsaturated fatty acids include hexadecatrienoic acid, alpha-linolenic acid, stearidonic acid, eicosatrienoic acid, eicosatetraenoic acid, eicosapentaenoic acid, heneicosapentaenoic acid, docosapentaenoic acid, docosahexaenoic acid, tetracosapentaenoic acid, tetracosahexaenoic acid, linoleic acid, gamma-linolenic acid, eicosadienoic acid, dihomo-gamma-linolenic acid, arachidonic acid, docosadienoic acid, adrenic acid, docosapentaenoic acid, tetracosatetraenoic acid, tetracosapentaenoic acid, oleic acid, eicosenoic acid, mead acid, erucic acid, nervonic acid, rumenic acid, α-calendic acid, β-calendic acid, jacaric acid, α-eleostearic acid, β-eleostearic acid, catalpic acid, punicic acid, rumelenic acid, α-parinaric acid, β-parinaric acid, bosseopentaenoic acid, pinolenic acid, podocarpic acid, palmitoleic acid, vaccenic acid, gadoleic acid, erucic acid, and combinations thereof.

Other non-limiting examples of hydrophobic acids include aromatic acids, such as 1-hydroxy-2-naphthoic acid, naphthalene-1,5-disulfonic acid, naphthalene-2-sulfonic acid, pamoic acid, cinnamic acid, phenylacetic acid, and combinations thereof.

In some embodiments, the hydrophobic acid may be a bile acid. Non-limiting examples of bile acids include chenodeoxycholic acid, ursodeoxycholic acid, deoxycholic acid, hycholic acid, beta-muricholic acid, cholic acid, an amino acid-

conjugated bile acid, and combinations thereof. An amino-acid conjugated bile acid may be conjugated to any suitable amino acid. In some embodiments, the amino acid-conjugated bile acid is a glycine-conjugated bile acid or a taurine-conjugated bile acid.

In some instances, a contemplated acid may have a molecular weight of less than about 1000 Da, in some embodiments less than about 500 Da, in some embodiments less than about 400 Da, in some embodiments less than about 300 Da, in some embodiments less than about 250 Da, in some embodiments less than about 200 Da, and in some embodiments less than about 150 Da. In some cases, the acid may have a molecular weight of between about 100 Da and about 1000 Da, in some embodiments between about 200 Da and about 800 Da, in some embodiments between about 200 Da and about 600 Da, in some embodiments between about 100 Da and about 300 Da, in some embodiments between about 200 Da and about 400 Da, and in some embodiments between about 300 Da and about 500 Da.

In some embodiments, a hydrophobic acid may be chosen, at least in part, on the basis of the strength of the acid. For example, the hydrophobic acid may have an acid dissociation constant in water (pK_a) of about -5 to about 7, in some embodiments about -3 to about 5, in some embodiments about -3 to about 4, in some embodiments about -3 to about 3.5, in some embodiments about -3 to about 3, in some embodiments about -3 to about 2, in some embodiments about -3 to about 1, in some embodiments about -3 to about 0.5, in some embodiments about -0.5 to about 0.5, in some embodiments about 1 to about 7, in some embodiments about 2 to about 7, in some embodiments about 3 to about 7, in some embodiments about 4 to about 6, in some embodiments about 4 to about 5.5, in some embodiments about 4 to about 5, and in some embodiments about 4.5 to about 5, determined at 25 °C. In some embodiments, the acid may have a pKa of less than about 7, less than about 5, less than about 3.5, less than about 3, less than about 2, less than about 1, or less than about 0, determined at 25 °C.

In some embodiments, a contemplated hydrophobic acid may have a phase transition temperature that is advantageous, for example, for improving the properties of the therapeutic nanoparticles in the final therapeutic nanoparticles. For instance, the acid may have a melting point of less than about 300 °C, in some cases less than about 100 °C, and in some casesless than about 50 °C. In certain embodiments, the acid may have a melting point of between about 5 °C and about 25 °C, in some cases between about 15 °C and about 50 °C, in some cases between about 30 °C and about

100 °C, in some cases between about 75 °C and about 150 °C, in some cases between about 125 °C and about 200 °C, in some cases between about 150 °C and about 250 °C, and in some cases between about 200 °C and about 300 °C. In some cases, the acid may have a melting point of less than about 15 °C, in some cases less than about 10 °C, or in some cases less than about 0 °C. In certain embodiments, the acid may have a melting point of between about -30 °C and about 0 °C or in some cases between about -20 °C and about -10 °C.

For example, an acid for use in methods and nanoparticles disclosed herein may be chosen, at least in part, on the basis of the solubility of the antibody therapeutic agent in a solvent comprising the acid. For example, in some embodiments, an anti-PD-1 antibody therapeutic agent dissolved in a solvent comprising the acid may have a solubility of between about 700 mg/mL to about 900 mg/mL, between about 600 mg/mL to about 800 mg/mL, between about 500 mg/mL to about 700 mg/mL to about 800 mg/mL, between about 15 mg/mL to about 200 mg/mL, between about 20 mg/mL to about 200 mg/mL, between about 25 mg/mL to about 200 mg/mL, between about 50 mg/mL to about 200 mg/mL, between about 75 mg/mL to about 200 mg/mL, between about 100 mg/mL to about 200 mg/mL, between about 125 mg/mL to about 175 mg/mL, between about 15 mg/mL to about 50 mg/mL, between about 25 mg/mL to about 75 mg/mL. In some embodiments, an antibody therapeutic agent dissolved in a solvent comprising the acid may have a solubility greater than about 10 mg/mL, greater than about 50 mg/mL, or greater than about 100 mg/mL. In some embodiments, an antibody therapeutic agent dissolved in a solvent comprising the hydrophobic acid (*e.g.,* a first solution consisting of the therapeutic agent, solvent, and hydrophobic acid) may have a solubility of at least about 2 times greater, in some embodiments at least about 5 times greater, in some embodiments at least about 10 times greater, in some embodiments at least about 20 times greater, in some embodiments about 2 times to about 20 times greater or in some embodiments about 10 times to about 20 times greater than when the antibody therapeutic agent is dissolved in a solvent that does not contain the hydrophobic acid (*e.g.,* a second solution consisting of the therapeutic agent and the solvent).

In some instances, the concentration of acid in a drug solution (i.e., an antibody therapeutic agent solution) may be between about 1 weight percent and about 30 weight percent, in some embodiments between about 2 weight percent and about 30 weight percent, in some embodiments between about 3 weight percent and about 30

weight percent, in some embodiments between about 4 weight percent and about 30 weight percent, in some embodiments between about 5 weight percent and about 30 weight percent, in some embodiments between about 6 weight percent and about 30 weight percent, in some embodiments between about 8 weight percent and about 30 weight percent, in some embodiments between about 10 weight percent and about 30 weight percent, in some embodiments between about 12 weight percent and about 30 weight percent, in some embodiments between about 14 weight percent and about 30 weight percent, in some embodiments between about 16 weight percent and about 30 weight percent, in some embodiments between about 1 weight percent and about 5 weight percent, in some embodiments between about 3 weight percent and about 9 weight percent, in some embodiments between about 6 weight percent and about 12 weight percent, in some embodiments between about 9 weight percent and about 15 weight percent, in some embodiments between about 12 weight percent and about 18 weight percent, and in some embodiments between about 15 weight percent and about 21 weight percent. In certain embodiments, the concentration of hydrophobic acid in a drug solution may be at least about 1 weight percent, in some embodiments at least about 2 weight percent, in some embodiments at least about 3 weight percent, in some embodiments at least about 5 weight percent, in some embodiments at least about 10 weight percent, in some embodiments at least about 15 weight percent, and in some embodiments at least about 20 weight percent.

In certain embodiments, the hydrophobic acid may have a solubility of less than about 2 g per 100 mL of water, in some embodiments less than about 1 g per 100 mL of water, in some embodiments less than about 100 mg per 100 mL of water, in some embodiments less than about 10 mg per 100 mL of water, and in some embodiments less than about 1 mg per 100 mL of water, determined at 25 °C. In other embodiments, the acid may have a solubility of between about 1 mg per 100 mL of water to about 2 g per 100 mL of water, in some embodiments between about 1 mg per 100 mL of water to about 1 g per 100 mL of water, in some embodiments between about 1 mg per 100 mL of water to about 500 mg per 100 mL of water, and in some embodiments between about 1 mg per 100 mL of water to about 100 mg per 100 mL of water, determined at 25 °C. In some embodiments, the hydrophobic acid may be essentially insoluble in water at 25 °C.

In some embodiments, disclosed nanoparticles may be essentially free of the hydrophobic acid used during the preparation of the nanoparticles. In other

embodiments, disclosed nanoparticles may comprise the hydrophobic acid. For instance, in some embodiments, the acid content in disclosed nanoparticles may be between about 0.05 weight percent to about 30 weight percent, in some embodiments between about 0.5 weight percent to about 30 weight percent, in some embodiments between about 1 weight percent to about 30 weight percent, in some embodiments between about 2 weight percent to about 30 weight percent, in some embodiments between about 3 weight percent to about 30 weight percent, in some embodiments between about 5 weight percent to about 30 weight percent, in some embodiments between about 7 weight percent to about 30 weight percent, in some embodiments between about 10 weight percent to about 30 weight percent, in some embodiments between about 15 weight percent to about 30 weight percent, in some embodiments between about 20 weight percent to about 30 weight percent, in some embodiments between about 0.05 weight percent to about 0.5 weight percent, in some embodiments between about 0.05 weight percent to about 5 weight percent, in some embodiments between about 1 weight percent to about 5 weight percent, in some embodiments between about 3 weight percent to about 10 weight percent, in some embodiments between about 5 weight percent to about 15 weight percent, and in some embodiments between about 10 weight percent to about 20 weight percent.

In some embodiments, disclosed nanoparticles substantially immediately release (*e.g.,* over about 1 minute to about 30 minutes, about 1 minute to about 25 minutes, about 5 minutes to about 30 minutes, about 5 minutes to about 1 hour, about 1 hour, or about 24 hours) less than about 2%, less than about 5%, less than about 10%, less than about 15%, less than about 20%, less than about 25%, less than about 30%, or less than 40% of the antibiotic therapeutic agent, for example when placed in a phosphate buffer solution at room temperature (*e.g.,* 25 °C) and/or at 37 °C. In certain embodiments, nanoparticles comprising a antibiotic therapeutic agent may release the antibiotic therapeutic agent when placed in an aqueous solution (*e.g.,* a phosphate buffer solution), *e.g.,* at 25 °C and/or at 37 °C, at a rate substantially corresponding to about 0.01 to about 50%, in some embodiments about 0.01 to about 25%, in some embodiments about 0.01 to about 15%, in some embodiments about 0.01 to about 10%, in some embodiments about 1 to about 40%, in some embodiments about 5 to about 40%, and in some embodiments about 10 to about 40% of the antibiotic therapeutic agent released over about 1 hour. In some embodiments, nanoparticles comprising a antibiotic therapeutic agent may release the antibiotic therapeutic agent

when placed in an aqueous solution (*e.g.,* a phosphate buffer solution), *e.g.,* at 25 °C and/or at 37 °C, at a rate substantially corresponding to about 10 to about 70%, in some embodiments about 10 to about 45%, in some embodiments about 10 to about 35%, or in some embodiments about 10 to about 25%, of the polymyxin/colistin antibiotic therapeutic agent released over about 4 hours.

In some embodiments, disclosed nanoparticles may substantially retain the antibody therapeutic agent, *e.g.,* for at least about 1 minute, at least about 1 hour, or more, when placed in a phosphate buffer solution at 37 °C.

In some embodiments, disclosed nanoparticles substantially release (*e.g.,* over about 1 minute to about 30 minutes, about 1 minute to about 25 minutes, about 5 minutes to about 30 minutes, about 5 minutes to about 1 hour, about 1 hour, or about 24 hours) less than about 2%, less than about 5%, less than about 10%, less than about 15%, less than about 20%, less than about 25%, less than about 30%, or less than 40% of the therapeutic agent, or the therapeutic agent - hydrophobic counter ion agent, such as an endo-lysosomal disrupting agent, the ion pair, for example when placed in a phosphate buffer solution at room temperature (*e.g.,* 25 °C) and/or at 37 °C. In certain embodiments, nanoparticles comprising a therapeutic agent may release the therapeutic agent when placed in an aqueous solution (*e.g.,* a phosphate buffer solution), *e.g.,* at 25 °C and/or at 37 °C, at a rate substantially corresponding to about 0.01 to about 50%, in some embodiments about 0.01 to about 25%, in some embodiments about 0.01 to about 15%, in some embodiments about 0.01 to about 10%, in some embodiments about 1 to about 40%, in some embodiments about 5 to about 40%, and in some embodiments about 10 to about 40% of the therapeutic agent released over about 1 hour. In some embodiments, nanoparticles comprising a therapeutic agent may release the therapeutic agent when placed in an aqueous solution (*e.g.,* a phosphate buffer solution), *e.g.,* at 25 °C and/or at 37 °C, at a rate substantially corresponding to about 10 to about 70%, in some embodiments about 10 to about 45%, in some embodiments about 10 to about 35%, or in some embodiments about 10 to about 25%, of the therapeutic agent released over about 4 hours.

In some embodiments, disclosed nanoparticles may substantially retain the therapeutic agent, *e.g.,* for at least about 1 minute, at least about 1 hour, or more, when placed in a phosphate buffer solution at 37 °C.

In some embodiments, the antibody therapeutic agent, or anti-PD-1 antibody is administered in conjuction with a therapeutic nanoparticle that encapsulates another

therapeutic agent. The second therapeutic agent may be encapsulated within the nanoparticle in addition to the antibody. In other embodiments, the second therapeutic agent is encapsulated within the nanoparticle and the antibody is attached to the nanoparticle, or attached to a ligand of the nanoparticle. In other embodiments, the therapeutic agent is selected from the group consisting of chemotherapeutic agents such as doxorubicin (adriamycin), mitoxantrone, gemcitabine (gemzar), daunorubicin, procarbazine, mitomycin, cytarabine, etoposide, methotrexate, 5- fluorouracil (5-FU), vinblastine, vincristine, bleomycin, paclitaxel (taxol), docetaxel (taxotere), aldesleukin, asparaginase, busulfan, carboplatin, cladribine, camptothecin, CPT-11, 10-hydroxy- 7-ethylcamptothecin (SN38), dacarbazine, S-I capecitabine, ftorafur, 5'deoxyflurouridine, UFT, eniluracil, deoxycytidine, 5-azacytosine, 5-azadeoxycytosine, allopurinol, 2-chloroadenosine, trimetrexate, aminopterin, methylene-10-deazaaminopterin (MDAM), oxaplatin, picoplatin, tetraplatin, satraplatin, platinum-DACH, ormaplatin, CI-973, JM-216, and analogs thereof, epirubicin, etoposide phosphate, 9- aminocamptothecin, 10,11-methylenedioxycamptothecin, karenitecin, 9-nitrocamptothecin, TAS 103, vindesine, L-phenylalanine mustard, ifosphamidemefosphamide, perfosfamide, trophosphamide carmustine, semustine, epothilones A-E, tomudex, 6-mercaptopurine, 6-thioguanine, amsacrine, etoposide phosphate, karenitecin, acyclovir, valacyclovir, ganciclovir, amantadine, rimantadine, lamivudine, zidovudine, bevacizumab, trastuzumab, rituximab, and 5-Fluorouracil, methotrexate, budesonide, sirolimus vincristine, and combinations thereof, or the therapeutic agent may be an siRNA.

In one embodiment, disclosed therapeutic nanoparticles may include a targeting ligand, *e.g.,* a low-molecular weight ligand. In certain embodiments, the low-molecular weight ligand is conjugated to a polymer, and the nanoparticle comprises a certain ratio of ligand- conjugated polymer (*e.g.,* PLA-PEG-Ligand) to non-functionalized polymer (*e.g.,* PLA-PEG or PLGA-PEG). The nanoparticle can have an optimized ratio of these two polymers such that an effective amount of ligand is associated with the nanoparticle for treatment of a disease or disorder, such as cancer. For example, an increased ligand density may increase target binding (cell binding/target uptake), making the nanoparticle "target specific." Alternatively, a certain concentration of non-functionalized polymer (*e.g.,* non-functionalized PLGA-PEG copolymer) in the nanoparticle can control inflammation and/or immunogenicity (*i.e.,* the ability to provoke an immune response), and allow the nanoparticle to have a circulation half-life that is adequate for the treatment of a disease or disorder. Furthermore, the non-functionalized polymer may,

in some embodiments, lower the rate of clearance from the circulatory system *via* the reticuloendothelial system (RES). Thus, the non-functionalized polymer may provide the nanoparticle with characteristics that may allow the particle to travel through the body upon administration. In some embodiments, a non-functionalized polymer may balance an otherwise high concentration of ligands, which can otherwise accelerate clearance by the subject, resulting in less delivery to the target cells.

In some embodiments, nanoparticles disclosed herein may include functionalized polymers conjugated to a ligand that constitute approximately 0.1 – 50, *e.g.,* 0.1 – 30, *e.g.,* 0.1 – 20, *e.g.*, 0.1 – 10 mole percent of the entire polymer composition of the nanoparticle (*i.e.,* functionalized + non-functionalized polymer). Also disclosed herein, in another embodiment, are nanoparticles that include a polymer conjugated (*e.g.,* covalently with (*i.e.,* through a linker (*e.g.,* an alkylene linker)) or a bond) with one or more low-molecular weight ligands, wherein the weight percent low-molecular weight ligand with respect to total polymer is between about 0.001 and 5, *e.g.,* between about 0.001 and 2, *e.g.,* between about 0.001 and 1.

In some embodiments, disclosed nanoparticles may be able to bind efficiently to or otherwise associate with a biological entity, for example, a particular membrane component or cell surface receptor. It should be appreciated that peptides, ligands, proteins, antibodies, or nanobodies can also be used to target the nanoparticles. Targeting of a therapeutic agent (*e.g.,* to a particular tissue or cell type, to a specific diseased tissue but not to normal tissue, *etc.*) is desirable for the treatment of tissue specific diseases such as solid tumor cancers (*e.g.,* prostate cancer). For example, in contrast to systemic delivery of a cytotoxic anti-cancer agent, the nanoparticles disclosed herein may substantially prevent the agent from killing healthy cells. Additionally, disclosed nanoparticles may allow for the administration of a lower dose of the agent (as compared to an effective amount of agent administered without disclosed nanoparticles or formulations) which may reduce the undesirable side effects commonly associated with traditional chemotherapy.

In general, a “nanoparticle” refers to any particle having a diameter of less than 1000 nm, *e.g.,* about 10 nm to about 200 nm. Disclosed therapeutic nanoparticles may include nanoparticles having a diameter of about 60 to about 120 nm, or about 70 to about 120 nm, or about 80 to about 120 nm, or about 90 to about 120 nm, or about 100 to about 120 nm, or about 60 to about 130 nm, or about 70 to about 130 nm, or about 80 to about 130 nm, or about 90 to about 130 nm, or about 100 to about 130 nm, or

about 110 to about 130 nm, or about 60 to about 140 nm, or about 70 to about 140 nm, or about 80 to about 140 nm, or about 90 to about 140 nm, or about 100 to about 140 nm, or about 110 to about 140 nm, or about 60 to about 150 nm, or about 70 to about 150 nm, or about 80 to about 150 nm, or about 90 to about 150 nm, or about 100 to about 150 nm, or about 110 to about 150 nm, or about 120 to about 150 nm. It should be appreciated that disclosed nanoparticles of the may be formed at a particular size, which mayetermine in uptake pathways, circulation time, targeting, internalization, and/or clearance.

Polymers

In some embodiments, the nanoparticles may comprise a matrix of polymers and a therapeutic agent, such as a therapeutic agent as described above, including an anti-PD-1 antibody, optionally together with a hydrophobic counter ion agent, *e.g.,* in a ion-pair with a hydrophobic counter ion agent such as an an endo-lysosomal disrupting agent. In some embodiments, a therapeutic agent and/or targeting moiety (*i.e.,* a low-molecular weight ligand) can be associated with at least part of the polymeric matrix. For example, in some embodiments, a targeting moiety (*e.g.,* ligand) can be covalently associated with the surface of a polymeric matrix. In some embodiments, covalent association is mediated by a linker. The therapeutic agent can be associated with the surface of, encapsulated within, surrounded by, and/or dispersed throughout the polymeric matrix.

A wide variety of polymers and methods for forming particles therefrom are known in the art of drug delivery. In some embodiments, the disclosure is directed toward nanoparticles with at least two macromolecules, wherein the first macromolecule comprises a first polymer bound to a low-molecular weight ligand (*e.g.,* targeting moiety); and the second macromolecule comprising a second polymer that is not bound to a targeting moiety. The nanoparticle can optionally include one or more additional, unfunctionalized, polymers.

Any suitable polymer can be used in the disclosed nanoparticles. Polymers can be natural or unnatural (synthetic) polymers. Polymers can be homopolymers or copolymers comprising two or more monomers. In terms of sequence, copolymers can be random, block, or comprise a combination of random and block sequences. Typically, polymers are organic polymers.

The term "polymer," as used herein, is given its ordinary meaning as used in the art, *i.e.,* a molecular structure comprising one or more repeat units (monomers), connected by covalent bonds. The repeat units may all be identical, or in some cases, there may be more than one type of repeat unit present within the polymer. In some cases, the polymer can be biologically derived, *i.e.,* a biopolymer. Non-limiting examples include peptides or proteins. In some cases, additional moieties may also be present in the polymer, for example biological moieties such as those described below. If more than one type of repeat unit is present within the polymer, then the polymer is said to be a "copolymer." It is to be understood that in any embodiment employing a polymer, the polymer being employed may be a copolymer in some cases. The repeat units forming the copolymer may be arranged in any fashion. For example, the repeat units may be arranged in a random order, in an alternating order, or as a block copolymer, *i.e.,* comprising one or more regions each comprising a first repeat unit (*e.g.,* a first block), and one or more regions each comprising a second repeat unit (*e.g.,* a second block), *etc.* Block copolymers may have two (a diblock copolymer), three (a triblock copolymer), or more numbers of distinct blocks.

Disclosed particles can include copolymers, which, in some embodiments, describes two or more polymers (such as those described herein) that have been associated with each other, usually by covalent bonding of the two or more polymers together. Thus, a copolymer may comprise a first polymer and a second polymer, which have been conjugated together to form a block copolymer where the first polymer can be a first block of the block copolymer and the second polymer can be a second block of the block copolymer. Of course, those of ordinary skill in the art will understand that a block copolymer may, in some cases, contain multiple blocks of polymer, and that a "block copolymer," as used herein, is not limited to only block copolymers having only a single first block and a single second block. For instance, a block copolymer may comprise a first block comprising a first polymer, a second block comprising a second polymer, and a third block comprising a third polymer or the first polymer, *etc.* In some cases, block copolymers can contain any number of first blocks of a first polymer and second blocks of a second polymer (and in certain cases, third blocks, fourth blocks, *etc.*). In addition, it should be noted that block copolymers can also be formed, in some instances, from other block copolymers. For example, a first block copolymer may be conjugated to another polymer (which may be a homopolymer, a biopolymer, another

block copolymer, *etc.*), to form a new block copolymer containing multiple types of blocks, and/or to other moieties (*e.g.,* to non-polymeric moieties).

In some embodiments, the polymer (*e.g.,* copolymer, *e.g.,* block copolymer) can be amphiphilic, *i.e.,* having a hydrophilic portion and a hydrophobic portion, or a relatively hydrophilic portion and a relatively hydrophobic portion. A hydrophilic polymer can be one generally that attracts water and a hydrophobic polymer can be one that generally repels water. A hydrophilic or a hydrophobic polymer can be identified, for example, by preparing a sample of the polymer and measuring its contact angle with water (typically, the polymer will have a contact angle of less than 60°, while a hydrophobic polymer will have a contact angle of greater than about 60°). In some cases, the hydrophilicity of two or more polymers may be measured relative to each other, *i.e.,* a first polymer may be more hydrophilic than a second polymer. For instance, the first polymer may have a smaller contact angle than the second polymer.

In one set of embodiments, a polymer (*e.g.,* copolymer, *e.g.,* block copolymer) contemplated herein includes a biocompatible polymer, *i.e.,* the polymer that does not typically induce an adverse response when inserted or injected into a living subject, for example, without significant inflammation and/or acute rejection of the polymer by the immune system, for instance, *via* a T-cell response. Accordingly, the therapeutic particles contemplated herein can be non-immunogenic. The term non-immunogenic as used herein refers to endogenous growth factor in its native state which normally elicits no, or only minimal levels of, circulating antibodies, T-cells, or reactive immune cells, and which normally does not elicit in the individual an immune response against itself.

Biocompatibility typically refers to the acute rejection of material by at least a portion of the immune system, *i.e.,* a nonbiocompatible material implanted into a subject provokes an immune response in the subject that can be severe enough such that the rejection of the material by the immune system cannot be adequately controlled, and often is of a degree such that the material must be removed from the subject. One simple test to determine biocompatibility can be to expose a polymer to cells *in vitro*; biocompatible polymers are polymers that typically will not result in significant cell death at moderate concentrations, *e.g.,* at concentrations of 50 micrograms/10^6 cells. For instance, a biocompatible polymer may cause less than about 20% cell death when exposed to cells such as fibroblasts or epithelial cells, even if phagocytosed or otherwise uptaken by such cells. Non-limiting examples of biocompatible polymers that may be useful in various embodiments include polydioxanone (PDO),

polyhydroxyalkanoate, polyhydroxybutyrate, poly(glycerol sebacate), polyglycolide (*i.e.,* poly(glycolic) acid) (PGA), polylactide (*i.e.,* poly(lactic) acid) (PLA), poly(lactic) acid-co-poly(glycolic) acid (PLGA), polycaprolactone, or copolymers or derivatives including these and/or other polymers.

In certain embodiments, contemplated biocompatible polymers may be biodegradable, *i.e.,* the polymer is able to degrade, chemically and/or biologically, within a physiological environment, such as within the body. As used herein, "biodegradable" polymers are those that, when introduced into cells, are broken down by the cellular machinery (biologically degradable) and/or by a chemical process, such as hydrolysis, (chemically degradable) into components that the cells can either reuse or dispose of without significant toxic effect on the cells. In one embodiment, the biodegradable polymer and their degradation byproducts can be biocompatible.

Particles disclosed herein may or may not contain PEG. In addition, certain embodiments can be directed towards copolymers containing poly(ester-ether)s, *e.g.,* polymers having repeat units joined by ester bonds (*e.g.,* R-C(O)-O-R' bonds) and ether bonds (*e.g.,* R-O- R' bonds). In some embodiments, a biodegradable polymer, such as a hydrolyzable polymer, containing carboxylic acid groups, may be conjugated with poly(ethylene glycol) repeat units to form a poly(ester-ether). A polymer (*e.g.,* copolymer, *e.g.,* block copolymer) containing poly(ethylene glycol) repeat units can also be referred to as a "PEGylated" polymer.

For instance, a contemplated polymer may be one that hydrolyzes spontaneously upon exposure to water (*e.g.,* within a subject), or the polymer may degrade upon exposure to heat (*e.g.,* at temperatures of about 37°C). Degradation of a polymer may occur at varying rates, depending on the polymer or copolymer used. For example, the half-life of the polymer (the time at which 50% of the polymer can be degraded into monomers and/or other nonpolymeric moieties) may be on the order of days, weeks, months, or years, depending on the polymer. The polymers may be biologically degraded, *e.g.,* by enzymatic activity or cellular machinery, in some cases, for example, through exposure to a lysozyme (*e.g.,* having relatively low pH). In some cases, the polymers may be broken down into monomers and/or other nonpolymeric moieties that cells can either reuse or dispose of without significant toxic effect on the cells (for example, polylactide may be hydrolyzed to form lactic acid, polyglycolide may be hydrolyzed to form glycolic acid, *etc.*).

In some embodiments, polymers may be polyesters, including 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; PEGylated polymers and copolymers of lactide and glycolide (*e.g.,* PEGylated PLA, PEGylated PGA, PEGylated PLGA, and derivatives thereof). In some embodiments, polyesters include, for example, polyanhydrides, poly(ortho ester) PEGylated poly(ortho ester), poly(caprolactone), PEGylated poly(caprolactone), polylysine, PEGylated polylysine, poly(ethylene imine), PEGylated poly(ethylene imine), 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.

In some embodiments, a polymer may be PLGA. PLGA is a biocompatible and biodegradable co-polymer of lactic acid and glycolic acid, and various forms of PLGA can be 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 can be 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. In some embodiments, the ratio of lactic acid to glycolic acid monomers in the polymer of the particle (*e.g.,* the PLGA block copolymer or PLGA-PEG block copolymer), may be selected to optimize for various parameters such as water uptake, therapeutic agent release and/or polymer degradation kinetics can be optimized.

In some embodiments, polymers may be one or more acrylic polymers. In certain embodiments, acrylic polymers include, for example, acrylic acid and methacrylic acid copolymers, methyl methacrylate copolymers, ethoxyethyl methacrylates, cyanoethyl methacrylate, amino alkyl methacrylate copolymer, poly(acrylic acid), poly(methacrylic acid), methacrylic acid alkylamide copolymer, poly(methyl methacrylate), poly(methacrylic acid polyacrylamide, amino alkyl methacrylate copolymer, glycidyl methacrylate copolymers, polycyanoacrylates, and combinations comprising one or more of the foregoing polymers. The acrylic polymer may comprise fully-polymerized

copolymers of acrylic and methacrylic acid esters with a low content of quaternary ammonium groups.

In some embodiments, polymers can be cationic polymers. In general, cationic polymers are able to condense and/or protect negatively charged strands of nucleic acids (*e.g.,* DNA, RNA, or derivatives thereof). Amine-containing polymers such as poly(lysine), polyethylene imine (PEI), and poly(amidoamine) dendrimers are contemplated for use, in some embodiments, in a disclosed particle.

In some embodiments, polymers can be degradable polyesters bearing cationic side chains. Examples of these polyesters include poly(L-lactide-co-L-lysine), poly(serine ester), poly(4-hydroxy-L-proline ester).

It is contemplated that PEG may be terminated and include an end group, for example, when PEG is not conjugated to a ligand. For example, PEG may terminate in a hydroxyl, a methoxy or other alkoxyl group, a methyl or other alkyl group, an aryl group, a carboxylic acid, an amine, an amide, an acetyl group, a guanidino group, or an imidazole. Other contemplated end groups include azide, alkyne, maleimide, aldehyde, hydrazide, hydroxylamine, alkoxyamine, or thiol moieties.

Those of ordinary skill in the art will know of methods and techniques for PEGylating a polymer, for example, by using EDC (l-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) and NHS (N-hydroxysuccinimide) to react a polymer to a PEG group terminating in an amine, by ring opening polymerization techniques (ROMP), or the like.

In one embodiment, the molecular weight (or *e.g.,* the ratio of molecular weights of, *e.g.,* different blocks of a copolymer) of the polymers can be optimized for effective treatment as disclosed herein. For example, the molecular weight of a polymer may influence particle degradation rate (such as when the molecular weight of a biodegradable polymer can be adjusted), solubility, water uptake, and drug release kinetics. For example, the molecular weight of the polymer (or *e.g.,* the ratio of molecular weights of, *e.g.,* different blocks of a copolymer) can be adjusted such that the particle biodegrades in the subject being treated within a reasonable period of time (ranging from a few hours to 1-2 weeks, 3-4 weeks, 5-6 weeks, 7-8 weeks, *etc.*).

A disclosed particle can for example comprise a diblock copolymer of PEG and PL(G)A, wherein for example, the PEG portion may have a number average molecular weight of about 1,000-20,000, *e.g.,* about 2,000-20,000, *e.g.,* about 2 to about 10,000, and the PL(G)A portion may have a number average molecular weight of about 5,000 to

about 20,000, or about 5,000-100,000, *e.g.,* about 20,000-70,000, *e.g.,* about 15,000-50,000.

For example, disclosed here is an exemplary therapeutic nanoparticle that includes about 10 to about 99 weight percent poly(lactic) acid-poly(ethylene)glycol copolymer or poly(lactic)-co-poly (glycolic) acid-poly(ethylene)glycol copolymer, or about 20 to about 80 weight percent, about 40 to about 80 weight percent, or about 30 to about 50 weight percent, or about 70 to about 90 weight percent poly(lactic) acid-poly(ethylene)glycol copolymer or poly(lactic)-co-poly (glycolic) acid-poly(ethylene)glycol copolymer. Exemplary poly(lactic) acid-poly(ethylene)glycol copolymers can include a number average molecular weight of about 10 to about 20kDa, about 15 to about 20 kDa, or about 10 to about 25 kDa of poly(lactic) acid and a number average molecular weight of about 4 to about 6, or about 2kDa to about 10 kDa of poly(ethylene)glycol.

In some embodiments, the poly(lactic) acid-poly(ethylene)glycol copolymer may have a poly(lactic) acid number average molecular weight fraction of about 0.6 to about 0.95, in some embodiments between about 0.7 to about 0.9, in some embodiments between about 0.6 to about 0.8, in some embodiments between about 0.7 to about 0.8, in some embodiments between about 0.75 to about 0.85, in some embodiments between about 0.8 to about 0.9, and in some embodiments between about 0.85 to about 0.95. It should be understood that the poly(lactic) acid number average molecular weight fraction may be calculated by dividing the number average molecular weight of the poly(lactic) acid component of the copolymer by the sum of the number average molecular weight of the poly(lactic) acid component and the number average molecular weight of the poly(ethylene)glycol component.

Disclosed nanoparticles may optionally include about 1 to about 50 weight percent poly(lactic) acid or poly(lactic) acid-co-poly (glycolic) acid (which does not include PEG), or may optionally include about 1 to about 50 weight percent, or about 10 to about 50 weight percent or about 30 to about 50 weight percent poly(lactic) acid or poly(lactic) acid-co-poly (glycolic) acid. For example, poly(lactic) or poly(lactic)-co-poly(glycolic) acid may have a number average molecule weight of about 5 to about 15 kDa, or about 5 to about 12 kDa. Exemplary PLA may have a number average molecular weight of about 5 to about 10 kDa. Exemplary PLGA may have a number average molecular weight of about 8 to about 12 kDa.

A therapeutic nanoparticle may, in some embodiments, contain about 10 to about 30 weight percent, in some embodiments about 10 to about 25 weight percent, in some embodiments about 10 to about 20 weight percent, in some embodiments about 10 to about 15 weight percent, in some embodiments about 15 to about 20 weight percent, in some embodiments about 15 to about 25 weight percent, in some embodiments about 20 to about 25 weight percent, in some embodiments about 20 to about 30 weight percent, or in some embodiments about 25 to about 30 weight percent of poly(ethylene)glycol, where the poly(ethylene)glycol may be present as a poly(lactic) acid-poly(ethylene)glycol copolymer, poly(lactic)-co-poly (glycolic) acid-poly(ethylene)glycol copolymer, or poly(ethylene)glycol homopolymer. In certain embodiments, the polymers of the nanoparticles can be conjugated to a lipid. The polymer can be, for example, a lipid-terminated PEG.

Targeting Moieties

Provided herein, in some embodiments, are nanoparticles that may include an optional targeting moiety, *i.e.,* a moiety able to bind to or otherwise associate with a biological entity, for example, a membrane component, a cell surface receptor, an antigen, or the like. A targeting moiety present on the surface of the particle may allow the particle to become localized at a particular targeting site, for instance, a tumor, a disease site, a tissue, an organ, a type of cell, *etc.* As such, the nanoparticle may then be "target specific." The drug or other payload may then, in some cases, be released from the particle and allowed to interact locally with the particular targeting site.

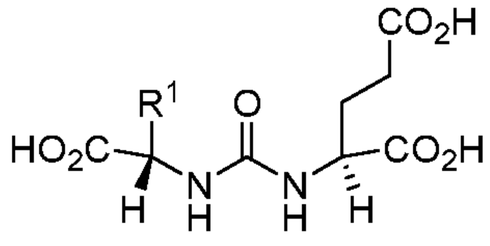
In one embodiment, a disclosed nanoparticle includes a targeting moiety that is a low-molecular weight ligand. The term "bind" or "binding," as used herein, refers to the interaction between a corresponding pair of molecules or portions thereof that exhibit mutual affinity or binding capacity, typically due to specific or non-specific binding or interaction, including, but not limited to, biochemical, physiological, and/or chemical interactions. "Biological binding" defines a type of interaction that occurs between pairs of molecules including proteins, nucleic acids, glycoproteins, carbohydrates, hormones, or the like. The term "binding partner" refers to a molecule that can undergo binding with a particular molecule. "Specific binding" refers to molecules, such as polynucleotides, that are able to bind to or recognize a binding partner (or a limited number of binding partners) to a substantially higher degree than to other, similar biological entities. In one set of embodiments, the targeting moiety has an affinity (as

measured *via* a disassociation constant) of less than about 1 micromolar, at least about 10 micromolar, or at least about 100 micromolar.

For example, a targeting portion may cause the particles to become localized to a tumor (*e.g.,* a solid tumor), a disease site, a tissue, an organ, a type of cell, *etc.* within the body of a subject, depending on the targeting moiety used. For example, a low-molecular weight ligand may become localized to a solid tumor, *e.g.,* breast or prostate tumors or cancer cells. The subject may be a human or non-human animal. Examples of subjects include, but are not limited to, a mammal such as a dog, a cat, a horse, a donkey, a rabbit, a cow, a pig, a sheep, a goat, a rat, a mouse, a guinea pig, a hamster, a primate, a human or the like.

Contemplated targeting moieties may include small molecules. In certain embodiments, the term "small molecule" refers to organic compounds, whether naturally- occurring or artificially created (*e.g., via* chemical synthesis) that have relatively low molecular weight and that are not proteins, polypeptides, or nucleic acids. Small molecules typically have multiple carbon-carbon bonds. In certain embodiments, small molecules are less than about 2000 g/mol in size. In some embodiments, small molecules are less than about 1500 g/mol or less than about 1000 g/mol. In some embodiments, small molecules are less than about 800 g/mol or less than about 500 g /mol, for example about 100 g/mol to about 600 g/mol, or about 200 g/mol to about 500 g/mol.

In some embodiments, the low-molecular weight ligand is of the Formulae I, II, III or IV:



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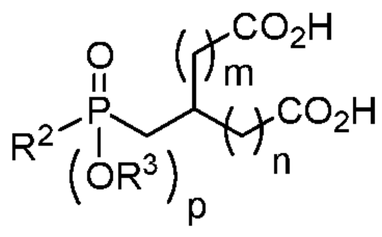

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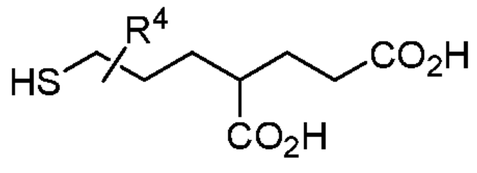

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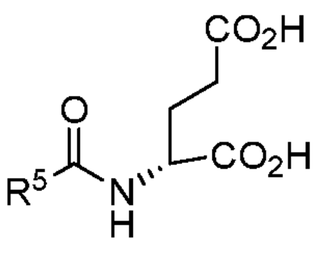


IV

and enantiomers, stereoisomers, rotamers, tautomers, diastereomers, or racemates thereof;

wherein m and n are each, independently, 0, 1, 2 or 3; p is 0 or 1;

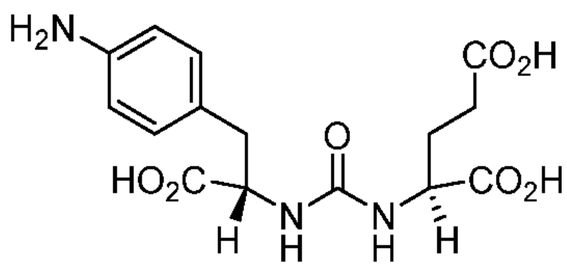
R^1, R^2, R^4, and R^5 are each, independently, selected from the group consisting of substituted or unsubstituted alkyl (*e.g.,* C_{1-10}-alkyl, C_{1-6}-alkyl, or C_{1-4}-alkyl), substituted

or unsubstituted aryl (*e.g.,* phenyl or pyridinyl), and any combination thereof; and R^3 is H or C_{1-6}- alkyl (*e.g.,* CH_3).

For compounds of Formulae I, II, III and IV, R^1, R^2, R^4 or R^5 comprise points of attachment to the nanoparticle, *e.g.,* a point of attachment to a polymer that forms part of a disclosed nanoparticle, *e.g.,* PEG. The point of attachment may be formed by a covalent bond, ionic bond, hydrogen bond, a bond formed by adsorption including chemical adsorption and physical adsorption, a bond formed from van der Waals bonds, or dispersion forces. For example, if R^1, R^2, R^4, or R^5 are defined as an aniline or C_{1-6}-alkyl-NH_2 group, any hydrogen (*e.g.,* an amino hydrogen) of these functional groups could be removed such that the low- molecular weight ligand is covalently bound to the polymeric matrix (*e.g.,* the PEG-block of the polymeric matrix) of the nanoparticle. As used herein, the term "covalent bond" refers to a bond between two atoms formed by sharing at least one pair of electrons.

In particular embodiments of the Formulae I, II, III or IV, R^1, R^2, R^4, and R^5 are each, independently, C_{1-6}-alkyl or phenyl, or any combination of C_{1-6}-alkyl or phenyl, which are independently substituted one or more times with OH, SH, NH_2, or CO_2H, and wherein the alkyl group may be interrupted by N(H), S, or O. In another embodiment, R^1, R^2, R4,and R^5 are each, independently, CH_2-Ph, $(CH_2)_2$-SH, CH_2-SH, $(CH_2)_2C(H)(NH_2)CO_2H$, $CH_2C(H)(NH_2)CO_2H$, $CH(NH_2)CH_2CO_2H$, $(CH_2)_2C(H)(SH)CO_2H$, CH_2-N(H)-Ph, O-CH_2-Ph, or O-$(CH_2)_2$-Ph, wherein each Ph may be independently substituted one or more times with OH, NH_2, CO_2H, or SH. For these formulae, the NH_2, OH or SH groups serve as the point of covalent attachment to the nanoparticle (*e.g.,* -N(H)-PEG, -O-PEG, or –S-PEG).

Exemplary ligands include: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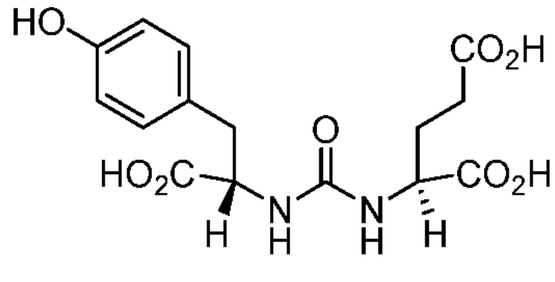
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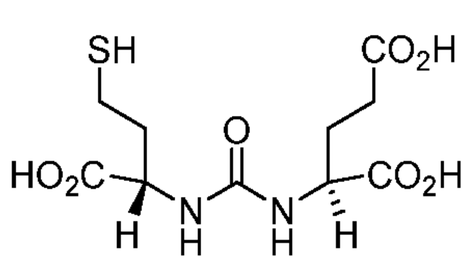
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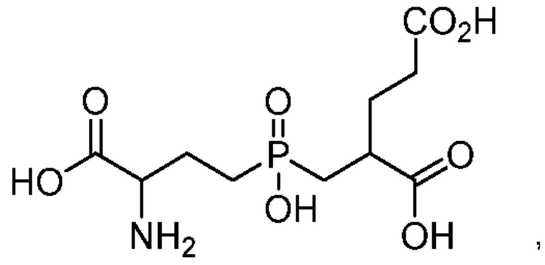
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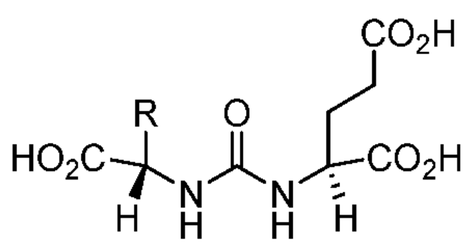
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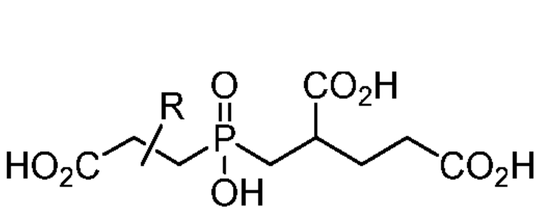
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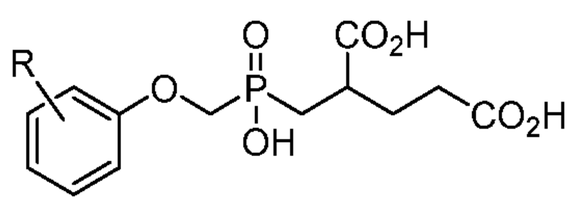
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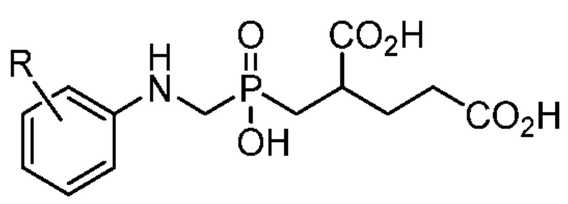
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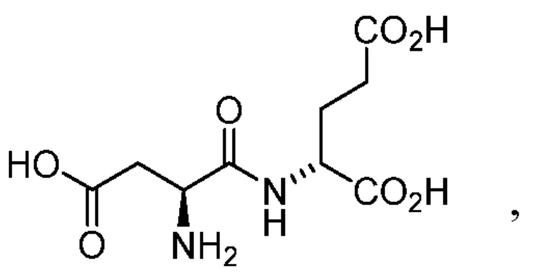
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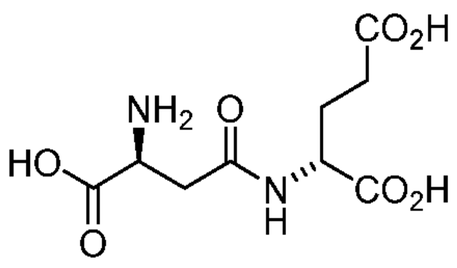
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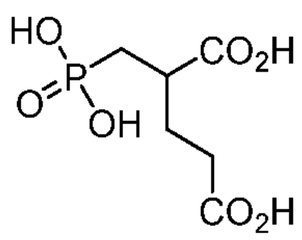
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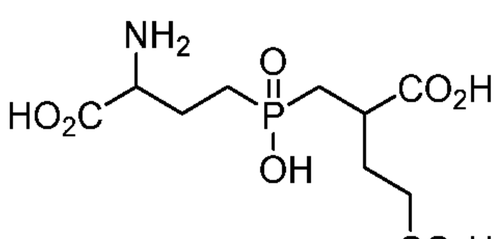
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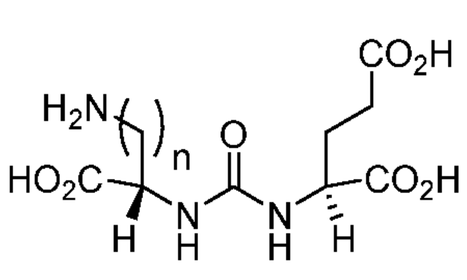
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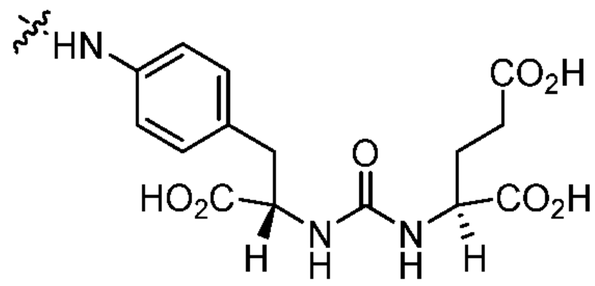
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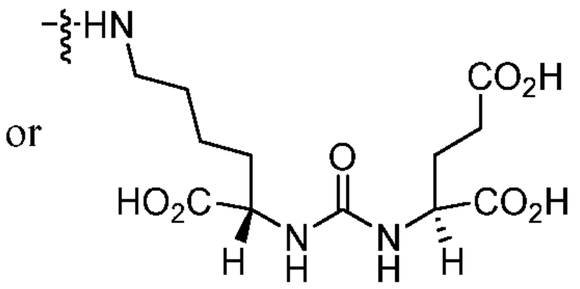
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and enantiomers, stereoisomers, rotamers, tautomers, diastereomers, or racemates thereof, wherein the NH_2, OH, or SH groups serve as the point of covalent attachment to the nanoparticle (*e.g.,* -N(H)-PEG, -O-PEG, or –S-PEG) or indicates the point of attachment to the nanoparticle, wherein n is 1, 2, 3, 4, 5, or 6, and wherein R is independently selected from the group consisting of NH_2, SH, OH, CO_2H, C_{1-6}-alkyl that is substituted with NH_2, SH, OH, or CO_2H, and phenyl that is substituted with NH_2, SH, OH, or CO_2H, and wherein R serves as the point of covalent attachment to the nanoparticle (*e.g.,* -N(H)-PEG, –S-PEG, -O-PEG, or CO_2- PEG). These compounds may be further substituted with NH_2, SH, OH, CO_2H, C_{1-6}-alkyl that is substituted with NH_2, SH, OH, or CO_2H, or phenyl that is substituted with NH_2, SH, OH or CO_2H,

wherein these functional groups can also serve as the point of covalent attachment to the nanoparticle.

In some embodiments, small molecule targeting moieties that may be used to target cells associated with solid tumors such as prostate or breast cancer tumors include PSMA peptidase inhibitors such as 2-PMPA, GPI5232, VA-033, phenylalkylphosphonamidates and/or analogs and derivatives thereof. In some embodiments, small molecule targeting moieties that may be used to target cells associated with prostate cancer tumors include thiol and indole thiol derivatives, such as 2-MPPA and 3-(2-mercaptoethyl)-1*H*-indole-2-carboxylic acid derivatives. In some embodiments, small molecule targeting moieties that may be used to target cells associated with prostate cancer tumors include hydroxamate derivatives. In some embodiments, small molecule targeting moieties that may be used to target cells associated with prostate cancer tumors include PBDA- and urea-based inhibitors, such as ZJ 43, ZJ 11, ZJ 17, ZJ 38 and/or and analogs and derivatives thereof, androgen receptor targeting agents (ARTAs), polyamines, such as putrescine, spermine, and spermidine, inhibitors of the enzyme glutamate carboxylase II (GCPII), also known as NAAG Peptidase or NAALADase.

In some embodiments, a contemplated ligand may be a small molecule DPPIV inhibitor that may target fibroblast activation proteins (FAP) for the treatment of solid tumors. Sulfonamides (Acetozolamide and others) ligands may target G250 antigens for the treatment of ccRCC (clear cell renal cell carcinoma) and other solid tumors. A ligand may comprises chlorotoxin that may target chlorotoxin receptors for the treatment of glioblastomas and solid tumors. Small molecules may target CXCR4 and matrix metalloproteinase (MMP) for the treatment of leukemia, lymphoma, and upregulation in angiogenesis.

In another embodiment, the targeting moiety can be a ligand that targets, folate receptor or toll receptors. In another embodiment, the targeting moiety is folate, folic acid, small molecules, antibodies, and nanobodies.

Targeting moieties can include a targeting antibody. Antibodies that target EpCAM (CD326), IGF-R, Mesothelin, Lewis-Y antigen (CD174), CanAg (MUC1, PEM, CA242, CD205), NCAM (CD56), Cripto, Melanotransferrin (P97), Glycoprotein NMB (CG56972), CD70 (CD27 Ligand), 5T4 (trophoblast glycoprotein), CD57, CD206, CD44, Carcinoembryonic antigen (CEA), GD2, CD40, Fibronectin ED-B, Endoglin (CD105), Tenascin C, Phosphatidylserine (PS), HER3, CD30, CD33, CD40, CD52, CD74,

CD138, CS1 (CD319,CRACC), TAG-72, CD2, CD64, ROBO4, DLL4, Tie2, and/or B7-H3 are contemplated. For example, Tenascin C may be targeted with a Tenascin C targeting antibody to treat gilomas and carcinomas. HER3 may be targeted with Heregulin or HER3 targeting antibodies to treat solid tumors. CD33 antibodies may target CD33 for treating AML. For example, antibodies targeting EpCAM (CD326), IGF-R, Mesothelin, Lewis-Y antigen (CD174), CanAg (MUC1, PEM, CA242, CD205), NCAM (CD56), and Cripto may be used for the treatment of solid tumors. Antibodies targeting Melanotransferrin (P97) may be used for treating primary and metastatic melanoma. CD30 may be targeted with antibodies for the treatment of Hodgkins and ALC lymphoma. CD74 may be targeted with antibodies for the treatment of multiple myeloma, NHL, or CLL. Affymax peptides may target TRAIL R2 for the treatment of solid tumors. Peptides such as Dyax Litt may target c-Met for the treating solid tumors. Other peptides and small molecule ligands may target EphA2 and EphB2 for the treatment of solid tumors.

For example, contemplated targeting moieties may include a nucleic acid, an aptamer, polypeptide, glycoprotein, carbohydrate, or lipid. For example, a targeting moiety can be a nucleic acid targeting moiety (*e.g.* an aptamer, *e.g.,* the A10 aptamer) that binds to a cell type specific marker. In general, an aptamer is an oligonucleotide (*e.g.,* DNA, RNA, or an analog or derivative thereof) that binds to a particular target, such as a polypeptide. In some embodiments, a targeting moiety may be a naturally occurring or synthetic ligand for a cell surface receptor, *e.g.,* a growth factor, hormone, LDL, transferrin, *etc.* A targeting moiety can be an antibody, which term is intended to include antibody fragments. Characteristic portions of antibodies, single chain targeting moieties can be identified, *e.g.,* using procedures such as phage display.

Targeting moieties may be a targeting peptide or targeting peptidomimetic that has a length of up to about 50 residues. For example, a targeting moiety may include the amino acid sequence AKERC, CREKA, ARYLQKLN, or AXYLZZLN, wherein X and Z are variable amino acids, or conservative variants or peptidomimetics thereof. In particular embodiments, the targeting moiety is a peptide that includes the amino acid sequence AKERC, CREKA, ARYLQKLN, or AXYLZZLN, wherein X and Z are variable amino acids, and has a length of less than 20, 50 or 100 residues. The CREKA (Cys Arg Glu Lys Ala) peptide or a peptidomimetic thereof or the octapeptide AXYLZZLN are also contemplated as targeting moieties, as well as peptides, or conservative variants or peptidomimetics thereof, that bind or form a complex with collagen IV, or that target

tissue basement membrane (*e.g.,* the basement membrane of a blood vessel). Exemplary targeting moieties include peptides that target ICAM (intercellular adhesion molecule, *e.g.,* ICAM-1). Other peptide based targeting moieties may be Affymax, Dyax Litt, YSA/SWL, NGR peptides and analogs with bestatin, Octreotide, CCK and Gastrin analogs, Leuprolide and analogs, GLP1/Exenatide, Lectin, and Mercator. It should be appreciated that the targeting ligands may target TRAIL R2, c-Met, EphA2, EphB2, Aminopeptidase N (CD13), VLA-4 (α4β1 integrin), CXCR4, Melanocortin receptor (MC1R), Somatostatin receptor, Cholecystokinin Receptor, GnRH Receptor, GLP1-receptor, E-Selectin, IL-11 receptor, Thrombospondin-1 receptor, Endostatin, CD79, and CD74.

Targeting moieties disclosed herein can be, in some embodiments, conjugated to a disclosed polymer or copolymer (*e.g.,* PLA-PEG), and such a polymer conjugate may form part of a disclosed nanoparticle.

In some embodiments, a therapeutic nanoparticle may include a polymer-drug conjugate. For example, a drug may be conjugated to a disclosed polymer or copolymer (*e.g.,* PLA-PEG), and such a polymer-drug conjugate may form part of a disclosed nanoparticle. For example, a disclosed therapeutic nanoparticle may optionally include about 0.2 to about 30 weight percent of a PLA-PEG or PLGA-PEG, wherein the PEG is functionalized with a drug (*e.g.,* PLA-PEG-Drug).

A disclosed polymeric conjugate (*e.g.,* a polymer-ligand conjugate) may be formed using any suitable conjugation technique. For instance, two compounds such as a targeting moiety or drug and a biocompatible polymer (*e.g.,* a biocompatible polymer and a poly(ethylene glycol)) may be conjugated together using techniques such as EDC-NHS chemistry (l-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride and *N*-hydroxysuccinimide) or a reaction involving a maleimide or a carboxylic acid, which can be conjugated to one end of a thiol, an amine, or a similarly functionalized polyether. The conjugation of a targeting moiety or drug and a polymer to form a polymer-targeting moiety conjugate or a polymer-drug conjugate can be performed in an organic solvent, such as, but not limited to, dichloromethane, acetonitrile, chloroform, dimethylformamide, tetrahydrofuran, acetone, or the like. Specific reaction conditions can be determined by those of ordinary skill in the art using no more than routine experimentation

In another set of embodiments, a conjugation reaction may be performed by reacting a polymer that comprises a carboxylic acid functional group (*e.g.,* a poly(ester-

ether) compound) with a polymer or other moiety (such as a targeting moiety or drug) comprising an amine. For instance, a targeting moiety, such as a low-molecular weight ligand, or a drug, such as dasatinib, may be reacted with an amine to form an amine-containing moiety, which can then be conjugated to the carboxylic acid of the polymer. Such a reaction may occur as a single-step reaction, *i.e.,* the conjugation is performed without using intermediates such as *N*-hydroxysuccinimide or a maleimide. In some embodiments, a drug may be reacted with an amine-containing linker to form an amine-containing drug, which can then be conjugated to the carboxylic acid of the polymer as described above. The conjugation reaction between the amine-containing moiety and the carboxylic acid-terminated polymer (such as a poly(ester-ether) compound) may be achieved, in one set of embodiments, by adding the amine-containing moiety, solubilized in an organic solvent such as (but not limited to) dichloromethane, acetonitrile, chloroform, tetrahydrofuran, acetone, formamide, dimethylformamide, pyridines, dioxane, or dimethylsulfoxide, to a solution containing the carboxylic acid-terminated polymer. The carboxylic acid-terminated polymer may be contained within an organic solvent such as, but not limited to, dichloromethane, acetonitrile, chloroform, dimethylformamide, tetrahydrofuran, or acetone. Reaction between the amine-containing moiety and the carboxylic acid-terminated polymer may occur spontaneously, in some cases. Unconjugated reactants may be washed away after such reactions, and the polymer may be precipitated in solvents such as, for instance, ethyl ether, hexane, methanol, or ethanol. In certain embodiments, a conjugate may be formed between an alcohol-containing moiety and carboxylic acid functional group of a polymer, which can be achieved similarly as described above for conjugates of amines and carboxylic acids.

It should be appreciated that in some embodiments, a nanoparticle may comprise two different type ligands. For example, a nanoparticle may comprise a small molecule ligand and a nucleic acid type ligand. In some embodiments, a nanoparticle may comprise three different type ligands. In some embodiments, a nanoparticle may comprise a multitude of different type ligands. It should be appreciated that a disclosed nanoparticle may include any number of different ligands.

Preparation of Nanoparticles

Another aspect of this disclosure is directed to systems and methods of making disclosed nanoparticles. In some embodiments, using two or more different polymers

(*e.g.,* copolymers, *e.g.,* block copolymers) in different ratios and producing particles from the polymers (*e.g.,* copolymers, *e.g.,* block copolymers), properties of the particles be controlled. For example, one polymer (*e.g.,* copolymer, *e.g.,* block copolymer) may include a low-molecular weight ligand, while another polymer (*e.g.,* copolymer, *e.g.,* block copolymer) may be chosen for its biocompatibility and/or its ability to control immunogenicity of the resultant particle.

Disclosed nanoparticles may be stable (*e.g.,* retain substantially all active agent) for example in a solution that may contain a saccharide, for at least about 3 days, about 4 days or at least about 5 days at room temperature, or at 25 °C.

In some embodiments, disclosed nanoparticles may also include a fatty alcohol, which may increase the rate of drug release. For example, disclosed nanoparticles may include a C_8-C_{30} alcohol such as cetyl alcohol, octanol, stearyl alcohol, arachidyl alcohol, docosonal, or octasonal.

Nanoparticles may have controlled release properties, *e.g.,* may be capable of delivering an amount of a polymyxin/colistin antibiotic therapeutic agent to a patient, *e.g.,* to specific site in a patient, over an extended period of time, *e.g.,* over 1 day, 1 week, or more.

In some embodiments, after administration to a subject or patient of a disclosed nanoparticle or a composition that includes a disclosed nanoparticle, the peak plasma concentration (C_{max}) of the polymyxin/colistin antibiotic therapeutic agent in the patient is substantially higher as compared to a C_{max} of the therapeutic agent if administered alone (*e.g.,* not as part of a nanoparticle).

In another embodiment, a disclosed nanoparticle including a therapeutic agent, when administered to a subject, may have a t_{max} of therapeutic agent substantially longer as compared to a t_{max} of the therapeutic agent administered alone.

Libraries of such particles may also be formed. For example, by varying the ratios of the two (or more) polymers within the particle, these libraries can be useful for screening tests, high-throughput assays, or the like. Entities within the library may vary by properties such as those described above, and in some cases, more than one property of the particles may be varied within the library. Accordingly, one embodiment is directed to a library of nanoparticles having different ratios of polymers with differing properties. The library may include any suitable ratio(s) of the polymers.

In some embodiments, the biocompatible polymer is a hydrophobic polymer. Non-limiting examples of biocompatible polymers include polylactide, polyglycolide, and/or poly(lactide-co-glycolide)

In a different embodiment, this disclosure provides for a nanoparticle comprising 1) a polymeric matrix; 2) optionally, an amphiphilic compound or layer that surrounds or is dispersed within the polymeric matrix forming a continuous or discontinuous shell for the particle; 3) a non-functionalized polymer that may form part of the polymeric matrix, and 4) optionally, a low molecular weight ligand that binds to a target protein conjugate such as PSMA, covalently attached to a polymer, which may form part of the polymeric matrix. For example, an amphiphilic layer may reduce water penetration into the nanoparticle, thereby enhancing drug encapsulation efficiency and slowing drug release.

As used herein, the term “amphiphilic” refers to a property where a molecule has both a polar portion and a non-polar portion. Often, an amphiphilic compound has a polar head attached to a long hydrophobic tail. In some embodiments, the polar portion is soluble in water, while the non-polar portion is insoluble in water. In addition, the polar portion may have either a formal positive charge, or a formal negative charge. Alternatively, the polar portion may have both a formal positive and a negative charge, and be a zwitterion or inner salt. In some embodiments, the amphiphilic compound can be, but is not limited to, one or a plurality of the following: naturally derived lipids, surfactants, or synthesized compounds with both hydrophilic and hydrophobic moieties.

Specific examples of amphiphilic compounds include, but are not limited to, phospholipids, such as 1,2 distearoyl-sn-glycero-3-phosphoethanolamine (DSPE), dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), diarachidoylphosphatidylcholine (DAPC), dibehenoylphosphatidylcholine (DBPC), ditricosanoylphosphatidylcholine (DTPC), and dilignoceroylphatidylcholine (DLPC), incorporated at a ratio of between 0.01-60 (weight lipid/w polymer), most preferably between 0.1-30 (weight lipid/w polymer). Phospholipids which may be used include, but are not limited to, phosphatidic acids, phosphatidyl cholines with both saturated and unsaturated lipids, phosphatidyl ethanolamines, phosphatidylglycerols, phosphatidylserines, phosphatidylinositols, lysophosphatidyl derivatives, cardiolipin, and β-acyl-y-alkyl phospholipids. Examples of phospholipids include, but are not limited to, phosphatidylcholines such as dioleoylphosphatidylcholine, dimyristoylphosphatidylcholine, dipentadecanoylphosphatidylcholine,

dilauroylphosphatidylcholine, dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), diarachidoylphosphatidylcholine (DAPC), dibehenoylphosphatidylcholine (DBPC), ditricosanoylphosphatidylcholine (DTPC), dilignoceroylphatidylcholine (DLPC); and phosphatidylethanolamines such as dioleoylphosphatidylethanolamine or 1-hexadecyl-2-palmitoylglycerophosphoethanolamine. Synthetic phospholipids with asymmetric acyl chains (*e.g.,* with one acyl chain of 6 carbons and another acyl chain of 12 carbons) may also be used.

In a particular embodiment, an amphiphilic component that can be used to form an amphiphilic layer is lecithin, and, in particular, phosphatidylcholine. Lecithin is an amphiphilic lipid and, as such, forms a phospholipid bilayer having the hydrophilic (polar) heads facing their surroundings, which are oftentimes aqueous, and the hydrophobic tails facing each other. Lecithin has an advantage of being a natural lipid that is available from, *e.g.,* soybean, and already has FDA approval for use in other delivery devices. In addition, a mixture of lipids such as lethicin is more advantageous than one single pure lipid.

In certain embodiments a disclosed nanoparticle has an amphiphilic monolayer, meaning the layer is not a phospholipid bilayer, but exists as a single continuous or discontinuous layer around, or within, the nanoparticle. The amphiphilic layer is "associated with" the nanoparticle, meaning it is positioned in some proximity to the polymeric matrix, such as surrounding the outside of the polymeric shell, or dispersed within the polymers that make up the nanoparticle.

In one set of embodiments, the particles are formed by providing a solution comprising one or more polymers, and contacting the solution with a polymer nonsolvent to produce the particle. The solution may be miscible or immiscible with the polymer nonsolvent. For example, a water-miscible liquid such as acetonitrile may contain the polymers, and particles are formed as the acetonitrile is contacted with water, a polymer nonsolvent, *e.g.,* by pouring the acetonitrile into the water at a controlled rate. The polymer contained within the solution, upon contact with the polymer nonsolvent, may then precipitate to form particles such as nanoparticles. Two liquids are said to be "immiscible" or not miscible, with each other when one is not soluble in the other to a level of at least 10% by weight at ambient temperature and pressure. Typically, an organic solution (*e.g.,* dichloromethane, acetonitrile, chloroform, tetrahydrofuran, acetone, formamide, dimethylformamide, pyridines, dioxane,

dimethylsulfoxide, *etc.*) and an aqueous liquid (*e.g.,* water, or water containing dissolved salts or other species, cell or biological media, ethanol, *etc.*) are immiscible with respect to each other. For example, the first solution may be poured into the second solution (at a suitable rate or speed). In some cases, particles such as nanoparticles may be formed as the first solution contacts the immiscible second liquid, *e.g.,* precipitation of the polymer upon contact causes the polymer to form nanoparticles while the first solution is poured into the second liquid, and in some cases, for example, when the rate of introduction is carefully controlled and kept at a relatively slow rate, nanoparticles may form. The control of such particle formation can be readily optimized by one of ordinary skill in the art using only routine experimentation.

Properties such as surface functionality, surface charge, size, zeta (ζ) potential, hydrophobicity, ability to control immunogenicity, and the like, may be highly controlled using a disclosed process. For instance, a library of particles may be synthesized, and screened to identify the particles having a particular ratio of polymers that allows the particles to have a specific density of moieties (*e.g.,* low-molecular weight ligands) present on the surface of the particle. This allows particles having one or more specific properties to be prepared, for example, a specific size and a specific surface density of moieties, without an undue degree of effort. Accordingly, certain embodiments are directed to screening techniques using such libraries, as well as any particles identified using such libraries. In addition, identification may occur by any suitable method. For instance, the identification may be direct or indirect, or proceed quantitatively or qualitatively.

In some embodiments, already-formed nanoparticles are functionalized with a targeting moiety using procedures analogous to those described for producing ligand-functionalized polymeric conjugates. For example, a first copolymer (PLGA-PEG, poly(lactide- co-glycolide) and poly(ethylene glycol)) is mixed with the therapeutic agent to form particles. The particles are then associated with a low-molecular weight ligand to form nanoparticles that can be used for the treatment of cancer. The particles can be associated with varying amounts of low-molecular weight ligands in order to control the ligand surface density of the nanoparticle, thereby altering the therapeutic characteristics of the nanoparticle. Furthermore, for example, by controlling parameters such as molecular weight, the molecular weight of PEG, and the nanoparticle surface charge, very precisely controlled particles may be obtained.

In another embodiment, a nanoemulsion process is provided, such as the process represented in FIGs. 1, 2A, and 2B. For example, a therapeutic agent (*e.g.,* dasatinib), a first polymer (for example, a diblock co-polymer such as PLA-PEG or PLGA-PEG, either of which may be optionally bound to a ligand) and an optional second polymer (*e.g.,* (PL(G)A-PEG or PLA), may be combined with an organic solution to form a first organic phase. Such first phase may include about 1 to about 50% weight solids, about 5 to about 50% weight solids, about 5 to about 40% weight solids, about 1 to about 15% weight solids, or about 10 to about 30% weight solids. The first organic phase may be combined with a first aqueous solution to form a second phase. The organic solution can include, for example, toluene, methyl ethyl ketone, acetonitrile, tetrahydrofuran, ethyl acetate, isopropyl alcohol, isopropyl acetate, dimethylformamide, methylene chloride, dichloromethane, chloroform, acetone, benzyl alcohol, Tween 80, Span 80, or the like, and combinations thereof. In an embodiment, the organic phase may include benzyl alcohol, ethyl acetate, and combinations thereof. The second phase can be between about 0.1 and 50 weight %, between about 1 and 50 weight %, between about 5 and 40 weight %, or between about 1 and 15 weight %, solids. The aqueous solution can be water, optionally in combination with one or more of sodium cholate, ethyl acetate, polyvinyl acetate and benzyl alcohol. In some embodiments, the pH of the aqueous phase may be selected based on the pK_a of the therapeutic agent and/or the pK_a of the hydrophobic counter ion agent, such as an endo- lysosomal disrupting agent.

For example, the oil or organic phase may use a solvent that is only partially miscible with the nonsolvent (water). Therefore, when mixed at a low enough ratio and/or when using water pre-saturated with the organic solvents, the oil phase remains liquid. The oil phase may be emulsified into an aqueous solution and, as liquid droplets, sheared into nanoparticles using, for example, high energy dispersion systems, such as homogenizers or sonicators. The aqueous portion of the emulsion, otherwise known as the “water phase”, may be surfactant solution consisting of sodium cholate and pre-saturated with ethyl acetate and benzyl alcohol. In some instances, the organic phase (*e.g.,* first organic phase) may include the therapeutic agent (*e.g.,* an antibody, such as an anti-PD-1 antibody). Additionally, in certain embodiments, the aqueous solution (*e.g.,* first aqueous solution) may include the substantially hydrophobic counter ion agent, such as an endo-lysosomal disrupting agent. In other embodiments, both the

therapeutic agent and the substantially hydrophobic counter ion agent, such as an endo- lysosomal disrupting agent, may be dissolved in the organic phase.

Emulsifying the second phase to form an emulsion phase may be performed, for example, in one or two emulsification steps. For example, a primary emulsion may be prepared, and then emulsified to form a fine emulsion. The primary emulsion can be formed, for example, using simple mixing, a high pressure homogenizer, probe sonicator, stir bar, or a rotor stator homogenizer. The primary emulsion may be formed into a fine emulsion through the use of *e.g.,* probe sonicator or a high pressure homogenizer, *e.g.*, by using 1, 2, 3, or more passes through a homogenizer. For example, when a high pressure homogenizer is used, the pressure used may be about 30 to about 60 psi, about 40 to about 50 psi, about 1000 to about 8000 psi, about 2000 to about 4000 psi, about 4000 to about 8000 psi, or about 4000 to about 5000 psi, *e.g.,* about 2000, 2500, 4000 or 5000 psi.

In some cases, fine emulsion conditions, which can be characterized by a very high surface to volume ratio of the droplets in the emulsion, can be chosen to maximize the solubility of the therapeutic agent. In certain embodiments, under fine emulsion conditions, equilibration of dissolved components can occur very quickly, *i.e.,* faster than solidification of the nanoparticles.

In some embodiments, the therapeutic agent may be combined in the second phase prior to emulsifying the second phase. In another example, the therapeutic agent may be dissolved in a separate miscible solution that is then fed into second phase during emulsification.

Either solvent evaporation or dilution may be needed to complete the extraction of the solvent and solidify the particles. For better control over the kinetics of extraction and a more scalable process, a solvent dilution via aqueous quench may be used. For example, the emulsion can be diluted into cold water to a concentration sufficient to dissolve all of the organic solvent to form a quenched phase. In some embodiments, quenching may be performed at least partially at a temperature of about 5 °C or less. For example, water used in the quenching may be at a temperature that is less that room temperature (*e.g.*, about 0 to about 10°C, or about 0 to about 5 °C). In certain embodiments, the quench may be chosen having a pH that is advantageous for quenching the emulsion phase, *e.g.,* by improving the properties of the nanoparticles, such as the release profile, or improving a nanoparticle parameter, such as the drug loading. The pH of the quench may be adjusted by acid or base titration, for example, or

by appropriate selection of a buffer. In some embodiments, the pH of the quench may be selected based on therapeutic agent.

In some embodiments, the pH of an aqueous solution used in a nanoparticle formulation process (*e.g.,* including, but not limited to, the aqueous phase, the emulsion phase, the quench, and the quenched phase) may be independently selected and may be between about 1 and about 3, in some embodiments between about 2 and about 4, in some embodiments between about 3 and about 5, in some embodiments between about 4 and about 6, in some embodiments between about 5 and about 7, in some embodiments between about 6 and about 8, in some embodiments between about 7 and about 9, and in some embodiments between about 8 and about 10. In certain embodiments, the pH of an aqueous solution used in a nanoparticle formulation process may be between about 3 and about 4, in some embodiments between about 4 and about 5, in some embodiments between about 5 and about 6, in some embodiments between about 6 and about 7, in some embodiments between about 7 and about 8, and in some embodiments between about 8 and about 9.

In some embodiments, not all of the therapeutic agent is encapsulated in the particles at this stage, and a drug solubilizer is added to the quenched phase to form a solubilized phase. The drug solubilizer may be for example, Tween 80, Tween 20, polyvinyl pyrrolidone, cyclodextran, sodium dodecyl sulfate, sodium cholate, diethylnitrosamine, sodium acetate, urea, glycerin, propylene glycol, glycofurol, poly(ethylene)glycol, bris(polyoxyethyleneglycolddodecyl ether, sodium benzoate, sodium salicylate, or combinations thereof. For example, Tween-80 may be added to the quenched nanoparticle suspension to solubilize the free drug and prevent the formation of drug crystals. In some embodiments, a ratio of drug solubilizer to the therapeutic agent molecules is about 200:1 to about 10:1, or in some embodiments about 100:1 to about 10:1.

The solubilized phase may be filtered to recover the nanoparticles. For example, ultrafiltration membranes may be used to concentrate the nanoparticle suspension and substantially eliminate organic solvent, free drug (*i.e.*, unencapsulated therapeutic agent), drug solubilizer, and other processing aids (surfactants). Exemplary filtration may be performed using a tangential flow filtration system. For example, by using a membrane with a pore size suitable to retain nanoparticles while allowing solutes, micelles, and organic solvent to pass, nanoparticles can be selectively separated.

Exemplary membranes with molecular weight cut- offs of about 300-500 kDa (~5-25 nm) may be used.

Diafiltration may be performed using a constant volume approach, meaning the diafiltrate (cold deionized water, *e.g.,* about 0 to about 5 °C, or 0 to about 10 °C) may added to the feed suspension at the same rate as the filtrate is removed from the suspension. In some embodiments, filtering may include a first filtering using a first temperature of about 0 to about 5 °C, or 0 to about 10 °C, and a second temperature of about 20 to about 30 °C, or 15 to about 35 °C. In some embodiments, filtering may include processing about 1 to about 30, in some cases about 1 to about 15, or in some cases 1 to about 6 diavolumes. For example, filtering may include processing about 1 to about 30, or in some cases about 1 to about 6 diavolumes, at about 0 to about 5 °C, and processing at least one diavolume (*e.g.,* about 1 to about 15, about 1 to about 3, or about 1 to about 2 diavolumes) at about 20 to about 30 °C. In some embodiments, filtering comprises processing different diavolumes at different distinct temperatures.

After purifying and concentrating the nanoparticle suspension, the particles may be passed through one, two or more sterilizing and/or depth filters, for example, using ~0.2 μm depth pre-filter. For example, a sterile filtration step may involve filtering the therapeutic nanoparticles using a filtration train at a controlled rate. In some embodiments, the filtration train may include a depth filter and a sterile filter.

In another embodiment of preparing nanoparticles, an organic phase is formed composed of a mixture of a therapeutic agent, and polymer (*e.g.,* a co-polymer, and optionally co-polymer with ligand). The organic phase is mixed with an aqueous phase at approximately a 1:5 ratio (oil phase:aqueous phase) where the aqueous phase is composed of a surfactant and some dissolved solvent. The primary emulsion is formed by the combination of the two phases under simple mixing or through the use of a rotor stator homogenizer. The primary emulsion is then formed into a fine emulsion through the use of a high pressure homogenizer. The fine emulsion is then quenched by addition to deionized water under mixing. In some embodiments, the quench:emulsion ratio may be about 2:1 to about 40:1, or in some embodiments about 5:1 to about 15:1. In some embodiments, the quench:emulsion ratio is approximately 8.5:1. Then a solution of Tween (*e.g.,* Tween 80) is added to the quench to achieve approximately 2% Tween overall. This serves to dissolve free, unencapsulated nucleicacid. The nanoparticles are then isolated through either centrifugation or ultrafiltration/diafiltration.

It will be appreciated that the amounts of polymer and therapeutic agent that are used in the preparation of the formulation may differ from a final formulation. For example, some of the therapeutic agent may not become completely incorporated in a nanoparticle and such free therapeutic agent be *e.g.*, filtered away. For example, in an embodiment, a first organic solution containing about 11 weight percent theoretical loading of therapeutic agent in a first organic solution, a second organic solution containing about 89 weight percent polymer (*e.g.*, the polymer may include about 2.5 mol percent of a targeting moiety conjugated to a polymer and about 97.5 mol percent PLA-PEG), may be used in the preparation of a formulation that results in, *e.g.,* a final nanoparticle comprising about 2 weight percent therapeutic agent, about 97.5 weight percent polymer (where the polymer may include about 1.25 mol percent of a targeting moiety conjugated to a polymer and about 98.75 mol percent PLA-PEG%). Such processes may provide final nanoparticles suitable for administration to a patient that includes about 1 to about 20 percent by weight therapeutic agent, *e.g.,* about 1, about 2, about 3, about 4, about 5, about 8, about 10, or about 15 percent therapeutic agent by weight.

Therapeutic Agents

In certain aspects of the invention, the therapeutic nanoparticles encapsulate, surround, or are connected or linked to an anti-PD-1 antibody, or antibodies that can act as agonists and/or antagonists of PD-1, thereby modulating immune responses regulated by PD-1. PD-1 is a 50-55 kDa type I transmembrane receptor that was originally identified in a T cell line undergoing activation-induced apoptosis. PD-1 is expressed on T cells, B cells, and macrophages. PD-1 is expressed on activated T cells, B cells, and monocytes. Experimental data implicates the interactions of PD-1 with its ligands in downregulation of central and peripheral immune responses. In particular, proliferation in wild-type T cells but not in PD-1-deficient T cells is inhibited in the presence of PD-L1.

The term “antibody,” as used in this disclosure, refers to an immunoglobulin or a fragment or a derivative thereof, and encompasses any polypeptide comprising an antigen- binding site, regardless whether it is produced in vitro or in vivo. The term includes, but is not limited to, polyclonal, monoclonal, monospecific, polyspecific, non-specific, humanized, single- chain, chimeric, synthetic, recombinant, hybrid, mutated, and grafted antibodies. The term antibody also includes antibody fragments such as

Fab, F(ab)2, Fv, scFv, Fd, dAb, and other antibody fragments that retain antigen-binding function, *i.e.,* the ability to bind PD-1 specifically. Typically, such fragments would comprise an antigen-binding domain.

In general, antibodies can be made, for example, using traditional hybridoma techniques (Kohler and Milstein (1975) Nature, 256: 495-499), recombinant DNA methods (U.S. Pat. No. 4,816,567), or phage display performed with antibody libraries (Clackson et al. (1991) Nature, 352: 624-628; Marks et al. (1991) J. Mol. Biol., 222: 581-597). For other antibody production techniques, see also Antibodies: A Laboratory Manual, eds. Harlow et al., Cold Spring Harbor Laboratory, 1988.

The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known in the art. Intact antibodies, also known as immunoglobulins, are typically tetrameric glycosylated proteins composed of two light (L) chains of approximately 25 kDa each and two heavy (H) chains of approximately 50 kDa each. Two types of light chain, designated as the λ chain and the κ chain, are found in antibodies. Depending on the amino acid sequence of the constant domain of heavy chains, immunoglobulins can be assigned to five major classes: A, D, E, G and M, and several of these may be further divided into subclasses (isotypes), *e.g.,* IgG1, IgG2, IgG3, IgG4, IgA1, and IgA2.

The therapeutic nanoparticles comprising anti-PD-1 antibodies are capable of modulating the PD-1-associated downregulation of the immune responses. The disclosed therapeutic nanoparticles comprising antibodies can act as either agonists or antagonists of PD-1, depending on the method of their use. The antibodies can be used to prevent, diagnose, or treat medical disorders in mammals, especially, in humans. Antibodies can also be used for isolating PD-1 or PD-1-expressing cells. Furthermore, the antibodies can be used to treat a subject at risk of or susceptible to a disorder or having a disorder associated with aberrant PD-1 expression or function.

In the cases of cancer outgrowth and viral infection, the activation of PD-1 signaling promotes immune tolerance, leading to the cancers or virus-infected cells escaping from immune surveillance and cancer metastasis or viral load increase Inhibition of PD-1 mediated cellular signaling by therapeutic agents can activate immune cells including T-cells, B-cells and NK cells, and therefore enhance immune cell functions inhibiting cancer cell growth or viral infection, and restore immune surveillance and immune memory function to treat such human diseases.

Pharmaceutical Formulations

Nanoparticles disclosed herein may be combined with pharmaceutically acceptable carriers to form a pharmaceutical composition, according to another aspect. As would be appreciated by one of skill in this art, the carriers may be chosen based on the route of administration as described below, the location of the target issue, the drug being delivered, the time course of delivery of the drug, *etc*.

The pharmaceutical compositions can be administered to a patient by any means known in the art including oral and parenteral routes. The term "patient," as used herein, refers to humans as well as non-humans, including, for example, mammals, birds, reptiles, amphibians, and fish. For instance, the non-humans may be mammals (*e.g.,* a rodent, a mouse, a rat, a rabbit, a monkey, a dog, a cat, a primate, or a pig). In certain embodiments parenteral routes are desirable since they avoid contact with the digestive enzymes that are found in the alimentary canal. According to such embodiments, inventive compositions may be administered by injection (*e.g.,* intravenous, subcutaneous or intramuscular, intraperitoneal injection), rectally, vaginally, topically (as by powders, creams, ointments, or drops), or by inhalation (as by sprays).

In a particular embodiment, the nanoparticles are administered to a subject in need thereof systemically, *e.g.,* by IV infusion or injection.

Injectable preparations, for example, sterile injectable aqueous or oleaginous suspensions may be formulated according to the known art using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation may also be a sterile injectable solution, suspension, or emulsion in a nontoxic parenterally acceptable diluent or solvent, for example, as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, U.S.P., and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil can be employed including synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid are used in the preparation of injectables. In one embodiment, the inventive conjugate is suspended in a carrier fluid comprising 1 % (w/v) sodium carboxymethyl cellulose and 0.1% (v/v) TWEEN™ 80. The injectable formulations can be sterilized, for example, by filtration through a bacteria-retaining filter, or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved or dispersed in sterile water or other sterile injectable medium prior to use

Solid dosage forms for oral administration include capsules, tablets, pills, powders, and granules. In such solid dosage forms, the encapsulated or unencapsulated conjugate is mixed with at least one inert, pharmaceutically acceptable excipient or carrier such as sodium citrate or dicalcium phosphate and/or (a) fillers or extenders such as starches, lactose, sucrose, glucose, mannitol, and silicic acid, (b) binders such as, for example, carboxymethylcellulose, alginates, gelatin, polyvinylpyrrolidinone, sucrose, and acacia, (c) humectants such as glycerol, (d) disintegrating agents such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate, (e) solution retarding agents such as paraffin, (f) absorption accelerators such as quaternary ammonium compounds, (g) wetting agents such as, for example, cetyl alcohol and glycerol monostearate, (h) absorbents such as kaolin and bentonite clay, and (i) lubricants such as talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, and mixtures thereof. In the case of capsules, tablets, and pills, the dosage form may also comprise buffering agents.

It will be appreciated that the exact dosage of a nanoparticle containing a therapeutic agent is chosen by the individual physician in view of the patient to be treated, in general, dosage and administration are adjusted to provide an effective amount of the therapeutic agent nanoparticle to the patient being treated. As used herein, the "effective amount" of a nanoparticle containing a therapeutic agent refers to the amount necessary to elicit the desired biological response. As will be appreciated by those of ordinary skill in this art, the effective amount of a nanoparticle containing a therapeutic agent may vary depending on such factors as the desired biological endpoint, the drug to be delivered, the target tissue, the route of administration, *etc*. For example, the effective amount of a nanoparticle containing a therapeutic agent might be the amount that results in a reduction in tumor size by a desired amount over a desired period of time. Additional factors which may be taken into account include the severity of the disease state; age, weight and gender of the patient being treated; diet, time and frequency of administration; drug combinations; reaction sensitivities; and tolerance/response to therapy.

The nanoparticles may be formulated in dosage unit form for ease of administration and uniformity of dosage. The expression “dosage unit form” as used herein refers to a physically discrete unit of nanoparticle appropriate for the patient to be treated. It will be understood, however, that the total daily usage of the compositions

will be decided by the attending physician within the scope of sound medical judgment. For any nanoparticle, the therapeutically effective dose can be estimated initially either in cell culture assays or in animal models, usually mice, rabbits, dogs, or pigs. The animal model is also used to achieve a desirable concentration range and route of administration. Such information can then be used to determine useful doses and routes for administration in humans. Therapeutic efficacy and toxicity of nanoparticles can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.,* ED_{50} (the dose is therapeutically effective in 50% of the population) and LD_{50} (the dose is lethal to 50% of the population). The dose ratio of toxic to therapeutic effects is the therapeutic index, and it can be expressed as the ratio, LD_{50}/ED_{50}. Pharmaceutical compositions which exhibit large therapeutic indices may be useful in some embodiments. The data obtained from cell culture assays and animal studies can be used in formulating a range of dosage for human use.

In an embodiment, compositions disclosed herein may include less than about 10 ppm of palladium, or less than about 8 ppm, or less than about 6 ppm of palladium. For example, provided here is a composition that includes nanoparticles having a polymeric conjugate wherein the composition has less than about 10 ppm of palladium.

In some embodiments, a composition suitable for freezing is contemplated, including nanoparticles disclosed herein and a solution suitable for freezing, *e.g.,* a sugar such as a mono, di, or poly saccharide, *e.g.,* sucrose and/or a trehalose, and/or a salt and/or a cyclodextrin solution is added to the nanoparticle suspension. The sugar (*e.g.,* sucrose or trehalose) may act, *e.g.,* as a cryoprotectant to prevent the particles from aggregating upon freezing. For example, provided herein is a nanoparticle formulation comprising a plurality of disclosed nanoparticles, sucrose, an ionic halide, and water; wherein the nanoparticles/sucrose/water/ionic halide is about 3-40%/10-40%/20-95%/0.1-10% (w/w/w/w) or about 5-10%/10-15%/80-90%/1-10% (w/w/w/w). For example, such solution may include nanoparticles as disclosed herein, about 5% to about 20% by weight sucrose and an ionic halide such as sodium chloride, in a concentration of about 10- 100 mM. In another example, provided herein is a nanoparticle formulation comprising a plurality of disclosed nanoparticles, trehalose, cyclodextrin, and water; wherein the nanoparticles/trehalose/water/cyclodextrin is about 3- 40%/1-25%/20-95%/1-25% (w/w/w/w) or about 5-10%/1-25%/80-90%/10-15% (w/w/w/w).

For example, a contemplated solution may include nanoparticles as disclosed

herein, about 1% to about 25% by weight of a disaccharide such as trehalose or sucrose (*e.g.,* about 5% to about 25% trehalose or sucrose, *e.g.,* about 10% trehalose or sucrose, or about 15% trehalose or sucrose, *e.g.,* about 5% sucrose) by weight) and a cyclodextrin such as β- cyclodextrin, in a concentration of about 1% to about 25% by weight (*e.g.,* about 5% to about 20%, *e.g.,* 10% or about 20% by weight, or about 15% to about 20% by weight cyclodextrin). Contemplated formulations may include a plurality of disclosed nanoparticles (*e.g.,* nanoparticles having PLA-PEG and an active agent), and about 2% to about 15 wt% (or about 4% to about 6wt%, *e.g.,* about 5wt%) sucrose and about 5wt% to about 20% (*e.g.,* about 7% wt percent to about 12 wt%, *e.g.,* about 10 wt%) of a cyclodextrin, *e.g.,* HPbCD).

The present disclosure relates in part to lyophilized pharmaceutical compositions that, when reconstituted, have a minimal amount of large aggregates. Such large aggregates may have a size greater than about 0.5 μm, greater than about 1 μm, or greater than about 10 μm, and can be undesirable in a reconstituted solution. Aggregate sizes can be measured using a variety of techniques including those indicated in the U.S. Pharmacopeia at 32 <788>, hereby incorporated by reference. The tests outlined in USP 32 <788> include a light obscuration particle count test, microscopic particle count test, laser diffraction, and single particle optical sensing. In one embodiment, the particle size in a given sample is measured using laser diffraction and/or single particle optical sensing.

The USP 32 <788> by light obscuration particle count test sets forth guidelines for sampling particle sizes in a suspension. For solutions with less than or equal to 100 mL, the preparation complies with the test if the average number of particles present does not exceed 6000 per container that are ≥10 μm and 600 per container that are ≥25 μm

As outlined in USP 32 <788>, the microscopic particle count test sets forth guidelines for determining particle amounts using a binocular microscope adjusted to 100 ± 10x magnification having an ocular micrometer. An ocular micrometer is a circular diameter graticule that consists of a circle divided into quadrants with black reference circles denoting 10 μm and 25 μm when viewed at 100x magnification. A linear scale is provided below the graticule. The number of particles with reference to 10 μm and 25 μm are visually tallied. For solutions with less than or equal to 100 mL, the preparation complies with the test if the average number of particles present does not exceed 3000 per container that are ≥10 μm and 300 per container that are ≥25 μm.

In some embodiments, a 10 mL aqueous sample of a disclosed composition upon reconstitution comprises less than 600 particles per ml having a size greater than or equal to 10 microns; and/or less than 60 particles per ml having a size greater than or equal to 25 microns.

Dynamic light scattering (DLS) may be used to measure particle size, but it relies on Brownian motion so the technique may not detect some larger particles. Laser diffraction relies on differences in the index of refraction between the particle and the suspension media. The technique is capable of detecting particles at the sub-micron to millimeter range. Relatively small (*e.g.,* about 1-5 weight %) amounts of larger particles can be determined in nanoparticle suspensions. Single particle optical sensing (SPOS) uses light obscuration of dilute suspensions to count individual particles of about 0.5 µm. By knowing the particle concentration of the measured sample, the weight percentage of aggregates or the aggregate concentration (particles/mL) can be calculated.

Formation of aggregates can occur during lyophilization due to the dehydration of the surface of the particles. This dehydration can be avoided by using lyoprotectants, such as disaccharides, in the suspension before lyophilization. Suitable disaccharides include sucrose, lactulose, lactose, maltose, trehalose, or cellobiose, and/or mixtures thereof. Other contemplated disaccharides include kojibiose, nigerose, isomaltose, β,β-trehalose, α,β-trehalose, sophorose, laminaribiose, gentiobiose, turanose, maltulose, palatinose, gentiobiulose, mannobiase, melibiose, melibiulose, rutinose, rutinulose, and xylobiose. Reconstitution shows equivalent DLS size distributions when compared to the starting suspension. However, laser diffraction can detect particles of >10 µm in size in some reconstituted solutions. Further, SPOS also may detect >10 µm sized particles at a concentration above that of the FDA guidelines (10^4-10^5 particles/mL for >10 µm particles).

In some embodiments, one or more ionic halide salts may be used as an additional lyoprotectant to a sugar, such as sucrose, trehalose or mixtures thereof. Sugars may include disaccharides, monosaccharides, trisaccharides, and/or polysaccharides, and may include other excipients, *e.g.,* glycerol and/or surfactants. Optionally, a cyclodextrin may be included as an additional lyoprotectant. The cyclodextrin may be added in place of the ionic halide salt. Alternatively, the cyclodextrin may be added in addition to the ionic halide salt.

Suitable ionic halide salts may include sodium chloride, calcium chloride, zinc chloride, or mixtures thereof. Additional suitable ionic halide salts include potassium chloride, magnesium chloride, ammonium chloride, sodium bromide, calcium bromide, zinc bromide, potassium bromide, magnesium bromide, ammonium bromide, sodium iodide, calcium iodide, zinc iodide, potassium iodide, magnesium iodide, or ammonium iodide, and/or mixtures thereof. In one embodiment, about 1 to about 15 weight percent sucrose may be used with an ionic halide salt. In one embodiment, the lyophilized pharmaceutical composition may comprise about 10 to about 100 mM sodium chloride. In another embodiment, the lyophilized pharmaceutical composition may comprise about 100 to about 500 mM of divalent ionic chloride salt, such as calcium chloride or zinc chloride. In yet another embodiment, the suspension to be lyophilized may further comprise a cyclodextrin, for example, about 1 to about 25 weight percent of cyclodextrin may be used.

A suitable cyclodextrin may include α-cyclodextrin, β-cyclodextrin, γ- cyclodextrin, or mixtures thereof. Exemplary cyclodextrins contemplated for use in the compositions disclosed herein include hydroxypropyl-β-cyclodextrin (HPbCD), hydroxyethyl-β-cyclodextrin, sulfobutylether-β-cyclodextrin, methyl-β -cyclodextrin, dimethyl-β -cyclodextrin, carboxymethyl-β-cyclodextrin, carboxymethyl ethyl -β-cyclodextrin, diethyl-β-cyclodextrin, tri- O-alkyl--β-cyclodextrin, glocosyl-β-cyclodextrin, and maltosyl-β-cyclodextrin. In one embodiment, about 1 to about 25 weight percent trehalose (*e.g.,* about 10% to about 15%, *e.g.,* 5 to about 20% by weight) may be used with cyclodextrin. In one embodiment, the lyophilized pharmaceutical composition may comprise about 1 to about 25 weight percent β -cyclodextrin. An exemplary composition may comprise nanoparticles comprising PLA-PEG, an active/therapeutic agent, about 4% to about 6% (*e.g.,* about 5% wt percent) sucrose, and about 8 to about 12 weight percent (*e.g.,* about 10 wt. %) HPbCD.

In one aspect, a lyophilized pharmaceutical composition is provided comprising disclosed nanoparticles, wherein upon reconstitution of the lyophilized pharmaceutical composition at a nanoparticle concentration of about 50 mg/mL, in less than or about 100 mL of an aqueous medium, the reconstituted composition suitable for parenteral administration comprises less than 6000, such as less than 3000, microparticles of greater than or equal to 10 microns; and/or less than 600, such as less than 300, microparticles of greater than or equal to 25 microns.

The number of microparticles can be determined by means such as the USP 32<788> by light obscuration particle count test, the USP 32 <788> by microscopic particle count test, laser diffraction, and single particle optical sensing.

In an aspect, a pharmaceutical composition suitable for parenteral use upon reconstitution is provided comprising a plurality of therapeutic particles each comprising a copolymer having a hydrophobic polymer segment and a hydrophilic polymer segment; an active agent; a sugar; and a cyclodextrin.

For example, the copolymer may be poly(lactic) acid-*block*-poly(ethylene)glycol copolymer. Upon reconstitution, a 100 mL aqueous sample may comprise less than 6000 particles having a size greater than or equal to 10 microns; and less than 600 particles having a size greater than or equal to 25 microns.

The step of adding a disaccharide and an ionic halide salt may comprise adding about 5 to about 15 weight percent sucrose or about 5 to about 20 weight percent trehalose (*e.g.,* about 10 to about 20 weight percent trehalose), and about 10 to about 500 mM ionic halide salt. The ionic halide salt may be selected from sodium chloride, calcium chloride, and zinc chloride, or mixtures thereof. In an embodiment, about 1 to about 25 weight percent cyclodextrin is also added.

In another embodiment, the step of adding a disaccharide and a cyclodextrin may comprise adding about 5 to about 15 weight percent sucrose or about 5 to about 20 weight percent trehalose (*e.g.,* about 10 to about 20 weight percent trehalose), and about 1 to about 25 weight percent cyclodextrin. In an embodiment, about 10 to about 15 weight percent cyclodextrin is added. The cyclodextrin may be selected from α-cyclodextrin, β-cyclodextrin, γ- cyclodextrin, or mixtures thereof.

In another aspect, a method of preventing substantial aggregation of particles in a pharmaceutical nanoparticle composition is provided comprising adding a sugar and a salt to the lyophilized formulation to prevent aggregation of the nanoparticles upon reconstitution. In an embodiment, a cyclodextrin is also added to the lyophilized formulation. In yet another aspect, a method of preventing substantial aggregation of particles in a pharmaceutical nanoparticle composition is provided comprising adding a sugar and a cyclodextrin to the lyophilized formulation to prevent aggregation of the nanoparticles upon reconstitution.

A contemplated lyophilized composition may have a therapeutic particle concentration of greater than about 40 mg/mL. The formulation suitable for parenteral administration may have less than about 600 particles having a size greater than 10

microns in a 10 mL dose. Lyophilizing may comprise freezing the composition at a temperature of greater than about -40 °C, or *e.g.,* less than about -30 °C, forming a frozen composition; and drying the frozen composition to form the lyophilized composition. The step of drying may occur at about 50 mTorr at a temperature of about -25 to about -34 °C, or about -30 to about -34 °C.

Methods of Treatment

In some embodiments, targeted nanoparticles may be used to treat, alleviate, ameliorate, relieve, delay onset of, inhibit progression of, reduce severity of, and/or reduce incidence of one or more symptoms or features of a disease, disorder, and/or condition. In some embodiments, targeted nanoparticles may be used to treat solid tumors, *e.g.,* cancer and/or cancer cells. In some embodiments, EGFR expressing cells are targeted. In some embodiments, solid tumors of other cancer cells expressing EGFR are targeted. In certain embodiments, targeted nanoparticles may be used to treat any cancer wherein PSMA is expressed on the surface of cancer cells or in the tumor neovasculature in a subject in need thereof, including the neovasculature of prostate or non-prostate solid tumors. Examples of the PSMA-related indication include, but are not limited to, prostate cancer, breast cancer, non-small cell lung cancer, colorectal carcinoma, and glioblastoma.

The term “cancer” includes pre-malignant as well as malignant cancers. Cancers include, but are not limited to, blood (*e.g.,* chronic myelogenous leukemia, chronic myelomonocytic leukemia, Philadelphia chromosome positive acute lymphoblastic leukemia, mantle cell lymphoma), prostate, gastric cancer, colorectal cancer, skin cancer, *e.g.,* melanomas or basal cell carcinomas, lung cancer (*e.g.*, non-small cell lung cancer), breast cancer, cancers of the head and neck, bronchus cancer, pancreatic cancer, urinary bladder cancer, brain or central nervous system cancer, peripheral nervous system cancer, esophageal cancer, cancer of the oral cavity or pharynx, liver cancer (*e.g.,* hepatocellular carcinoma), kidney cancer (*e.g.,* renal cell carcinoma), testicular cancer, biliary tract cancer, small bowel or appendix cancer, gastrointestinal stromal tumor, salivary gland cancer, thyroid gland cancer, adrenal gland cancer, osteosarcoma, chondrosarcoma, cancer of hematological tissues, and the like. “Cancer cells” can be in the form of a tumor (*i.e.,* a solid tumor), exist alone within a subject (*e.g.,* leukemia cells), or be cell lines derived from a cancer.

In some embodiments of the invention, the therapeutic nanoparticles containing anti-PD-1 antibodies are used in the treatment of squamous non small cell lung cancer. In certain embodiments, a “therapeutically effective amount” of an inventive targeted particle is that amount effective for treating, alleviating, ameliorating, relieving, delaying onset of, inhibiting progression of, reducing severity of, and/or reducing incidence of one or more symptoms or features of squamous non small cell lung cancer.

Cancer can be associated with a variety of physical symptoms. Symptoms of cancer generally depend on the type and location of the tumor. For example, lung cancer can cause coughing, shortness of breath, and chest pain, while colon cancer often causes diarrhea, constipation, and blood in the stool. However, to give but a few examples, the following symptoms are often generally associated with many cancers: fever, chills, night sweats, cough, dyspnea, weight loss, loss of appetite, anorexia, nausea, vomiting, diarrhea, anemia, jaundice, hepatomegaly, hemoptysis, fatigue, malaise, cognitive dysfunction, depression, hormonal disturbances, neutropenia, pain, non-healing sores, enlarged lymph nodes, peripheral neuropathy, and sexual dysfunction.

In one aspect, a method for the treatment of cancer (*e.g.,* leukemia) is provided. It should be appreciated that that other methods of treatments, such as infection, inflammation, genetic disorders, *etc.,* can be accomplished as disclosed herein. In some embodiments, the treatment of cancer comprises administering a therapeutically effective amount of inventive targeted particles to a subject in need thereof, in such amounts and for such time as is necessary to achieve the desired result. In certain embodiments, a “therapeutically effective amount” of an inventive targeted particle is that amount effective for treating, alleviating, ameliorating, relieving, delaying onset of, inhibiting progression of, reducing severity of, and/or reducing incidence of one or more symptoms or features of cancer.

In one aspect, a method for administering inventive compositions to a subject suffering from cancer (*e.g.,* leukemia) is provided. In some embodiments, particles may be administered to a subject in such amounts and for such time as is necessary to achieve the desired result (*i.e.,* treatment of cancer). In certain embodiments, a “therapeutically effective amount” of an inventive targeted particle is that amount effective for treating, alleviating, ameliorating, relieving, delaying onset of, inhibiting progression of, reducing severity of, and/or reducing incidence of one or more symptoms or features of cancer.

Inventive therapeutic protocols involve administering a therapeutically effective amount of an inventive targeted particle to a healthy individual (*i.e.,* a subject who does not display any symptoms of cancer and/or who has not been diagnosed with cancer). For example, healthy individuals may be "immunized" with an inventive targeted particle prior to development of cancer and/or onset of symptoms of cancer; at risk individuals (*e.g.,* patients who have a family history of cancer; patients carrying one or more genetic mutations associated with development of cancer; patients having a genetic polymorphism associated with development of cancer; patients infected by a virus associated with development of cancer; patients with habits and/or lifestyles associated with development of cancer; *etc.*) can be treated substantially contemporaneously with (*e.g.,* within 48 hours, within 24 hours, or within 12 hours of) the onset of symptoms of cancer. Of course individuals known to have cancer may receive inventive treatment at any time.

In other embodiments, disclosed nanoparticles can be used to inhibit the growth of cancer cells, *e.g.,* myelogenous leukemia cancer cells. As used herein, the term "inhibits growth of cancer cells" or "inhibiting growth of cancer cells" refers to any slowing of the rate of cancer cell proliferation and/or migration, arrest of cancer cell proliferation and/or migration, or killing of cancer cells, such that the rate of cancer cell growth is reduced in comparison with the observed or predicted rate of growth of an untreated control cancer cell. The term "inhibits growth" can also refer to a reduction in size or disappearance of a cancer cell or tumor, as well as to a reduction in its metastatic potential. Preferably, such an inhibition at the cellular level may reduce the size, deter the growth, reduce the aggressiveness, or prevent or inhibit metastasis of a cancer in a patient. Those skilled in the art can readily determine, by any of a variety of suitable indicia, whether cancer cell growth is inhibited

Inhibition of cancer cell growth may be evidenced, for example, by arrest of cancer cells in a particular phase of the cell cycle, *e.g.,* arrest at the G2/M phase of the cell cycle. Inhibition of cancer cell growth can also be evidenced by direct or indirect measurement of cancer cell or tumor size. In human cancer patients, such measurements generally are made using well known imaging methods such as magnetic resonance imaging, computerized axial tomography and X-rays. Cancer cell growth can also be determined indirectly, such as by determining the levels of circulating carcinoembryonic antigen, prostate specific antigen or other cancer-specific antigens that are correlated with cancer cell growth. Inhibition of cancer growth is also

generally correlated with prolonged survival and/or increased health and well-being of the subject.

In some embodiments, the therapeutic nanoparticles are administered side-by-side, or are coadministered with another therapeutic agent, such as an anti-PD-1 antibody.

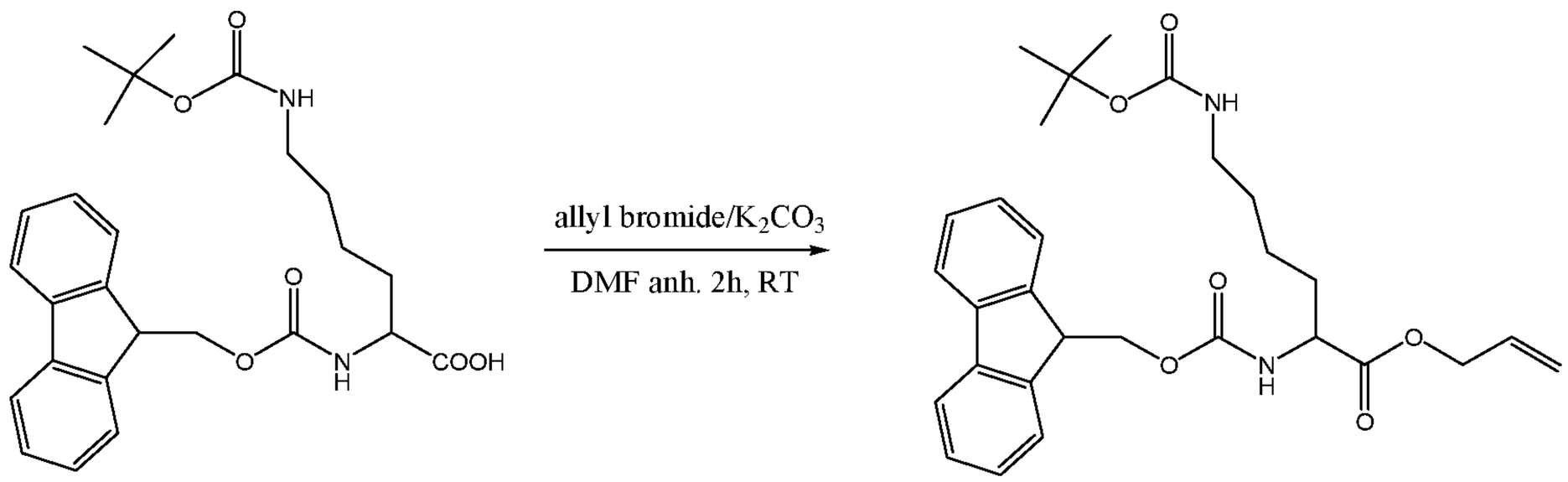
Also provided herein are methods of administering to a patient a nanoparticle disclosed herein including an active agent, wherein, upon administration to a patient, such nanoparticles substantially reduces the volume of distribution and/or substantially reduces free C_{max}, as compared to administration of the agent alone (*i.e.,* not as a disclosed nanoparticle).

U.S. Patent No. 8,206,747, issued June 26, 2012, entitled "Drug Loaded Polymeric Nanoparticles and Methods of Making and Using Same" is hereby incorporated by reference in its entirety.

EXAMPLES

The invention now being generally described, it will be more readily understood by reference to the following examples which are included merely for purposes of illustration of certain aspects and embodiments of the present invention, and are not intended to limit the invention in any way.

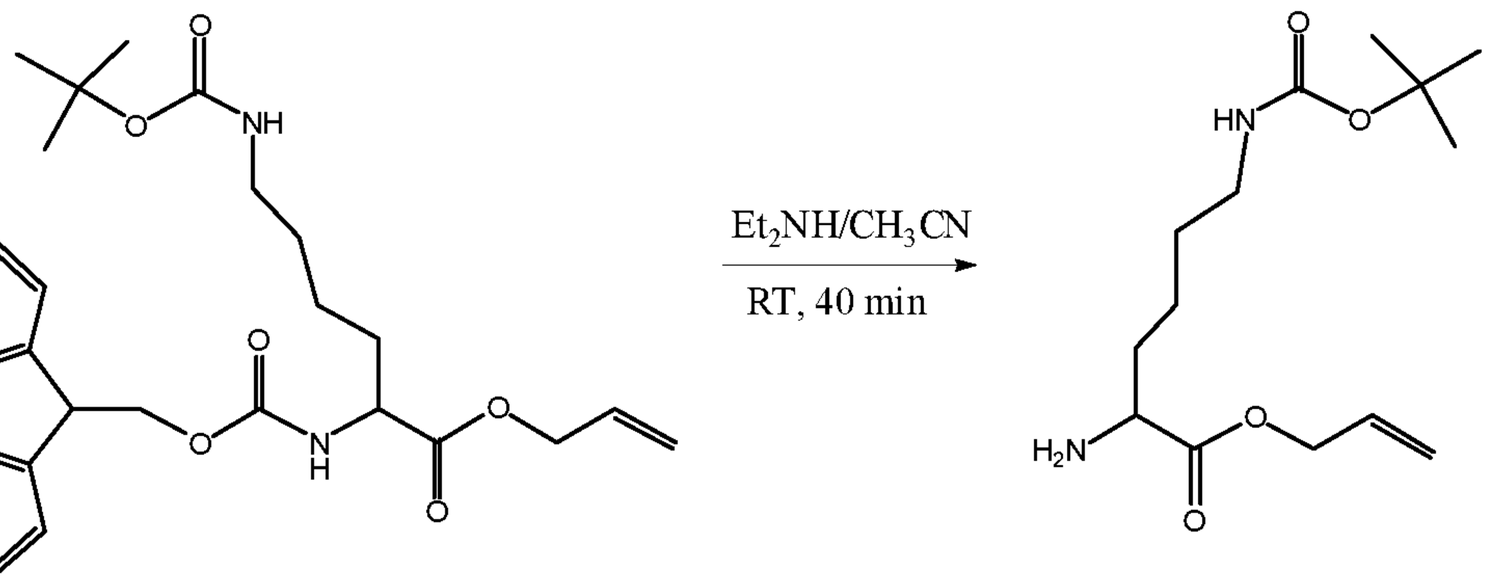
EXAMPLE 1: Synthesis of a Low-Molecular Weight PSMA Ligand (GL2)



FW 468 FW 508

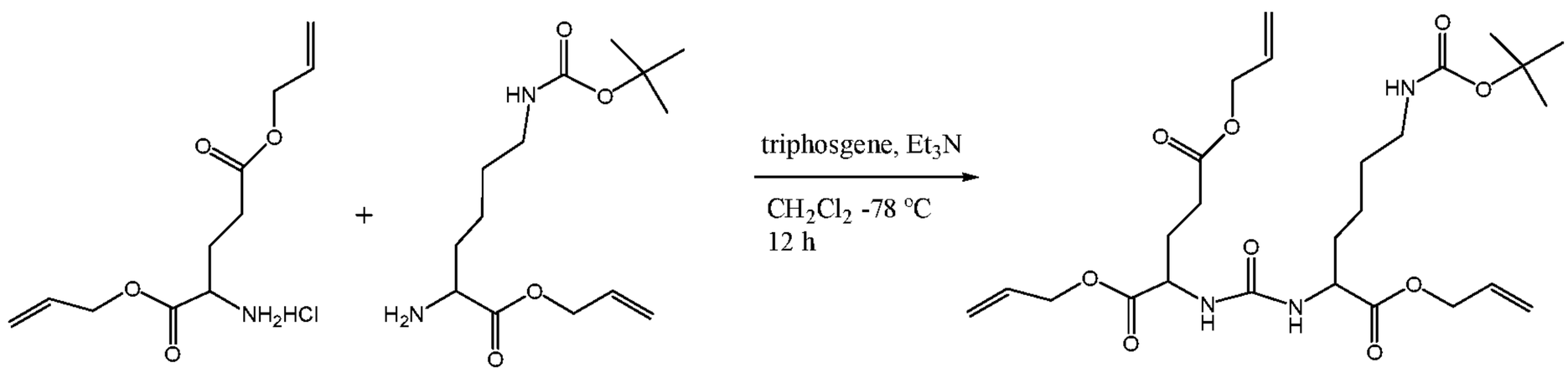
5 g (10.67 mmol) of the starting compound was dissolved in 150 mL of anhydrous DMF. To this solution was added allyl bromide (6.3 mL, 72 mmol) and K_2CO_3 (1.47 g, 10.67 mmol). The reaction was stirred for 2 h, the solvent was removed,

the crude material was dissolved in AcOEt and washed with H_2O until pH neutral. The organic phase was dried with $MgSO_4$ (anhydrous) and evaporated to give 5.15 g (95%) of material. (TLC in CH_2Cl_2:MeOH 20:1 Rf = 0.9, started compound Rf = 0.1, revealed with ninhydrin and uv light).



FW 508 FW 286

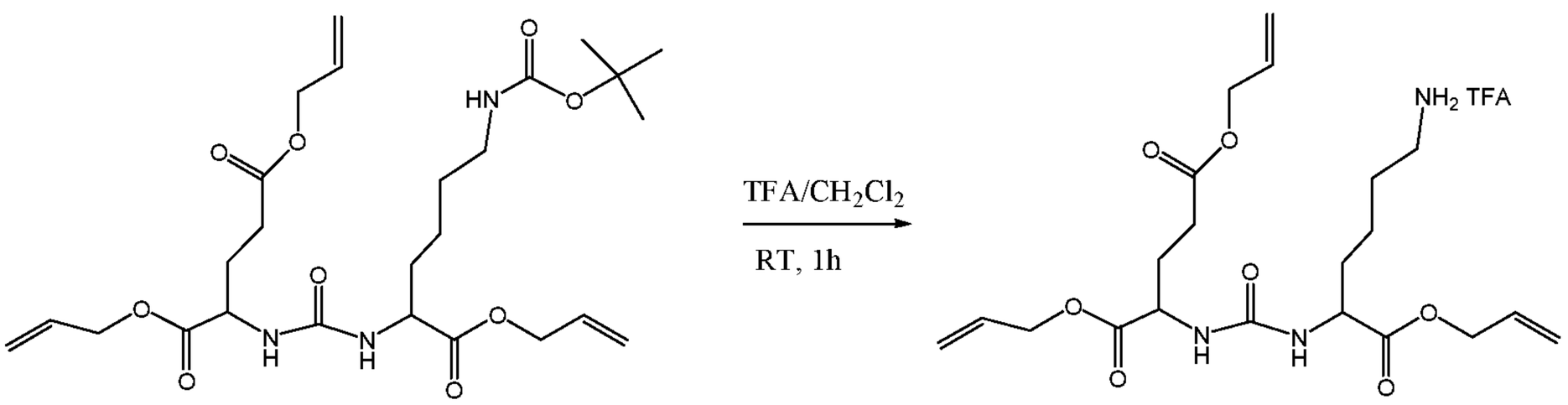
To a solution of the compound (5.15 g, 10.13 mmol) in CH_3CN (50 mL) was added Et_2NH (20 mL, 0.19 mol). The reaction was stirred at room temperature for 40 min. The solvent was removed and the compound was purified by column chromatography (Hexane:AcOEt 3:2) to give 2.6 g (90%). (TLC in CH_2Cl_2:MeOH 10:1 Rf = 0.4, revealed with ninhydrin (the compound has a violet color). ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 5.95-5.85 (m, 1H, - CH_2CHCH_2), 5.36-5.24 (m, 2H, -CH_2CHCH_2), 4.62-4.60 (m, 3H, -CH_2CHCH_2, N*H*Boc), 3.46 (t, 1H, C*H*(Lys)), 3.11-3.07 (m, 2H, CH_2NHBoc), 1.79 (bs, 2H, NH_2), 1.79-1.43 (m, 6H,3CH_2(Lys)), 1.43 (s, 9H, Boc).



FW 263.7 FW 286 FW 539

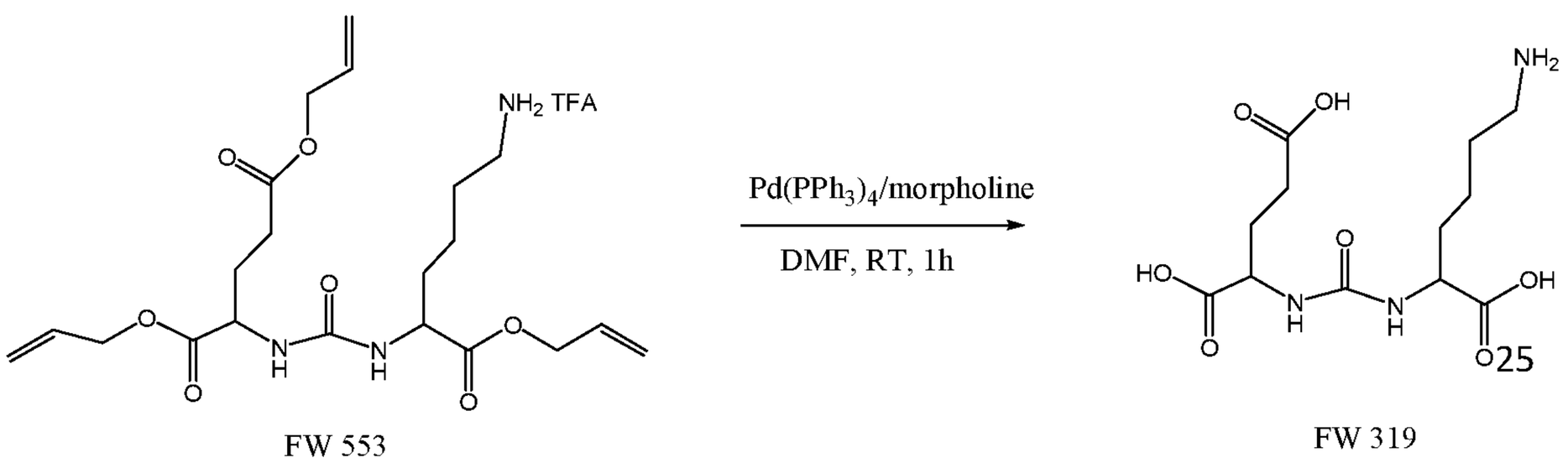
To a stirred solution of diallyl glutamate (3.96 g, 15 mmol) and triphosgene (1.47 g, 4.95 mmol) in CH_2Cl_2 (143 mL) at -78 ºC was added Et_3N (6.4 mL, 46 mmol) in CH_2Cl_2 (28 mL). The reaction mixture was allowed to warm to room temperature and

stirred for 1.5 h. The Lysine derivative (2.6 g, 9.09 mmol) in a solution of CH_2Cl_2 (36 mL) was then added at -78 ºC and the reaction was stirred at room temperature for 12 h. The solution was diluted with CH_2Cl_2, washed twice with H_2O, dried over $MgSO_4$ (anh.) and purified by column chromatography (Hexane:AcOEt 3:1→2:1→AcOEt) to give 4 g (82%) (TLC in CH_2Cl_2:MeOH 20:1 Rf = 0.3, revealed with ninhydrin). ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 5.97-5.84 (m, 3H, 3-CH_2CHCH_2), 5.50 (bt, 2H, 2N*H*urea), 5.36-5.20 (m, 6H, 3-CH_2CHCH_2), 4.81 (bs, 1H, N*H*Boc), 4.68-4.40 (m, 8H,3-CH_2CHCH_2, C*H*(Lys), C*H*(glu)), 3.09-3.05 (m, 2H, CH_2NHBoc), 2.52-2.39 (m, 2H, C*H*2(glu.)), 2.25-2.14 and 2.02-1.92 (2m, 2H, CH_2(glu.)), 1.87-1.64 (m, 4H, 2CH_2(Lys)), 1.51- 1.35 (m, 2H, CH_2(Lys)), 1.44 (s, 9H, Boc).



FW 539 FW 553

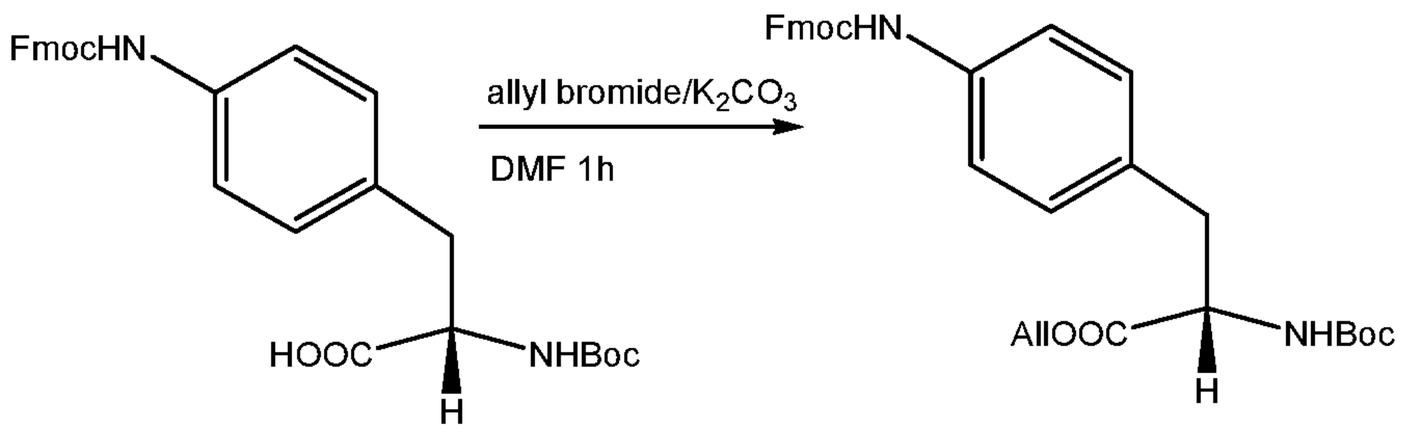
To a solution of the compound (4 g, 7.42 mmol) in dry CH_2Cl_2 (40 mL) was added at 0 ºC TFA (9 mL). The reaction was stirred at room temperature for 1 h. The solvent was removed under vacuum until complete dryness, to give 4.1 g (quantitative). (TLC in CH_2Cl_2:MeOH 20:1 Rf = 0.1, revealed with ninhydrin). ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 6.27-6.16 (2d, 2H, 2N*H*urea), 5.96-5.82 (m, 3H, 3-CH_2CHCH_2), 5.35-5.20 (m, 6H, 3-CH_2CHCH_2), 4.61-4.55 (m, 6H, 3-CH_2CHCH_2), 4.46-4.41 (m, 2H, C*H*(Lys), C*H*(glu)), 2.99 (m, 2H, CH_2NHBoc), 2.46 (m, 2H, CH_2(glu.)), 2.23-2.11 and 2.01-1.88 2H, CH_2(glu.)), 1.88-1.67 (m, 4H, 2CH_2(Lys)), 1.45 (m, 2H, CH_2(Lys)).



FW 553 FW 319

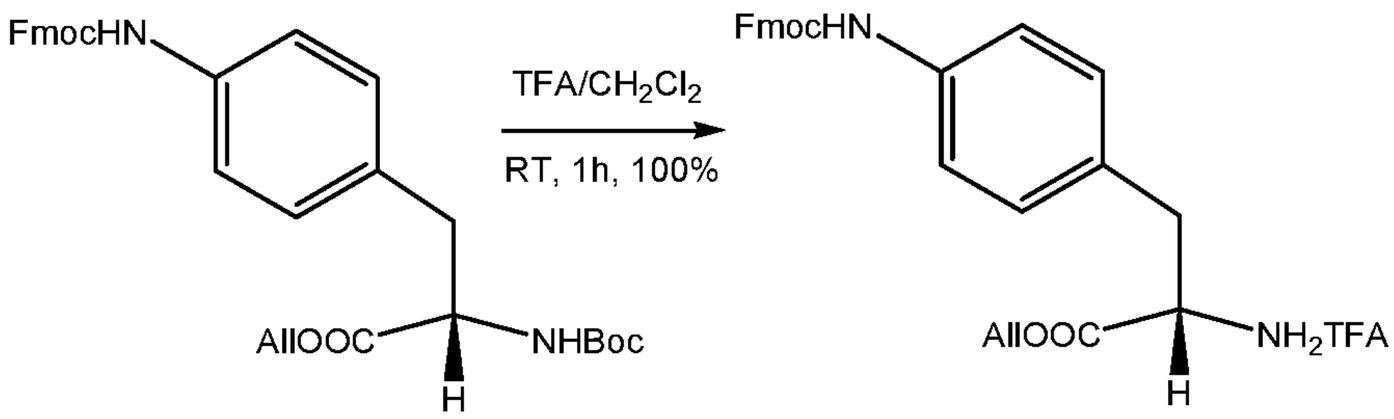
To a solution of the compound (2 g, 3.6 mmol) in DMF (anh.) (62 mL) under argon was added $Pd(PPh_3)_4$ (0.7 g, 0.6 mmol) and morpholine (5.4 mL, 60.7 mmol) at 0 ºC. The reaction was stirred at room temperature for 1 h. The solvent was removed. The crude product was washed twice with CH_2Cl_2, and then solved in H_2O. To this solution was added a diluted solution of NaOH (0.01 N) until the pH was very basic. The solvent was removed under reduced pressure. The solid was washed again with CH_2Cl_2, AcOEt, and a mixture of MeOH-CH_2Cl_2 (1:1), solved in H_2O and neutralized with Amberlite IR-120 H^+ resin. The solvent was evaporated, and the compound was precipitated with MeOH, to give 1 g (87 %) of GL2. ^{1}H-NMR (D_2O, 300 MHz) δ 4.07 (m, 2H, C*H*(Lys), C*H*(glu)), 2.98 (m, 2H, C$H_2$$NH_2$), 2.36 (m, 2H, C$H_2$(glu.)), 2.08-2.00 (m, 1H, CH_2(glu)), 1.93-1.60 (m, 5H, CH_2(glu.), 2CH_2(Lys)), 1.41 (m, 2H, CH_2(Lys)). Mass ESI: 320.47 [M + H^+], 342.42 [M + Na^+].

EXAMPLE 2: Synthesis of a Low-Molecular Weight PSMA Ligand (GL1)

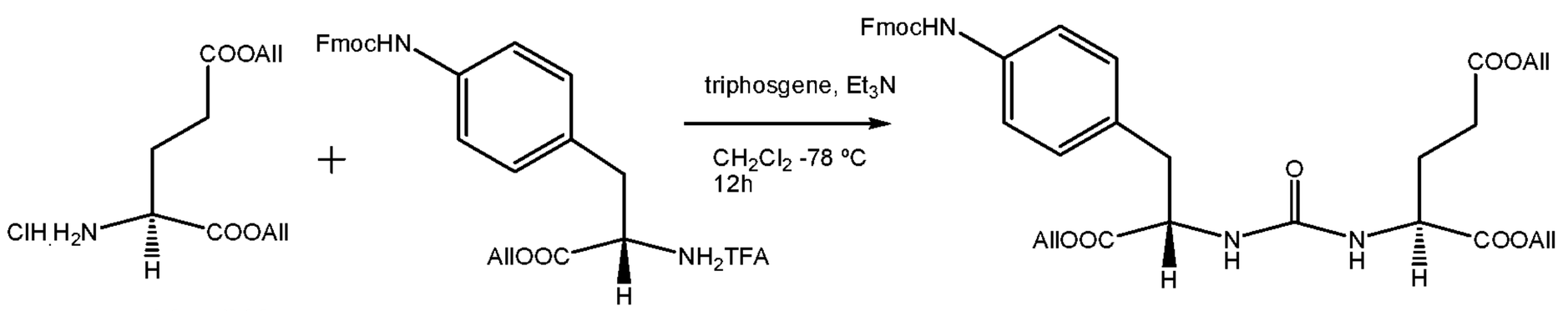


commercial available

130 mg (0.258 mmol) of the starting compound was dissolved in 3 mL of DMF (anh.) To this solution was added allyl bromide (150 µ L, 1.72 mmol) and K_2CO_3 (41 mg, 0.3 mmol). The reaction was stirred for 1 h, the solvent was removed, the crude product was dissolved in AcOEt and washed with H_2O until pH neutral. The organic phase was dried with $MgSO_4$ (anh.) and evaporated to give 130 mg (93%). (TLC in CH_2Cl_2:MeOH 20:1 Rf = 0.9, started compound Rf = 0.1, revealed with ninhydrin and uv light).^{1}H-NMR ($CDCl_3$, 300 MHz) δ 7.81-7.05 (12H, aromatics), 6.81 (bs, 1H, N*H*Fmoc), 5.93-5.81 (m, 1H, -CH_2C*H*CH_2), 5.35-5.24 (m, 2H, -CH_2CHCH_2), 5.00 (bd, 1H, N*H*boc), 4.61-4.53 (m, 5H, -CH_2CHCH_2, CH_2(Fmoc) C*H*(pheala.)), 4.28 (t, 1H, C*H*(Fmoc)), 3.12-2.98 (m, 2H, CH_2(pheala.), 1.44 (s, 9H, Boc).

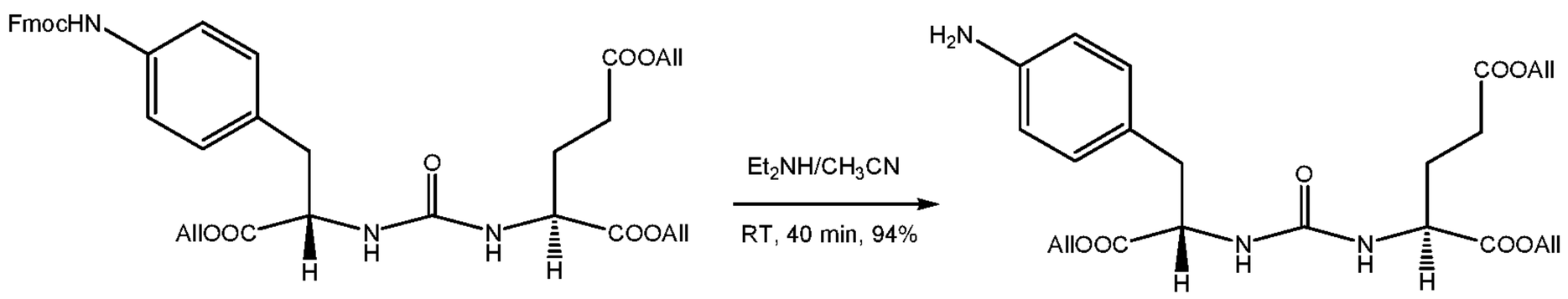


To a solution of the compound (120 mg, 0.221 mmol) in dry CH_2Cl_2 (2 mL) was added at 0 ºC TFA (1 mL). The reaction was stirred at room temperature for 1 h. The solvent was removed under vacuum, water was added and removed again, CH_2Cl_2 was added and removed again until complete dryness to give 120 mg (quantitative). (TLC in CH_2Cl_2:MeOH 20:1 Rf = 0.1, revealed with ninhydrin and uv light). ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 7.80-7.00 (13H, aromatics, N*H*Fmoc), 5.90-5.75 (m, 1H, -CH_2CHCH_2), 5.35-5.19 (m, 3H, -CH_2CHCH_2, N*H*boc), 4.70-4.40 (2m, 5H, -CH_2CHCH_2, C*H*$_2$(Fmoc), C*H*(pheala.)), 4.20 (t, 1H, C*H*(Fmoc)), 3.40-3.05 (m, 2H, C*H*$_2$(pheala.)).

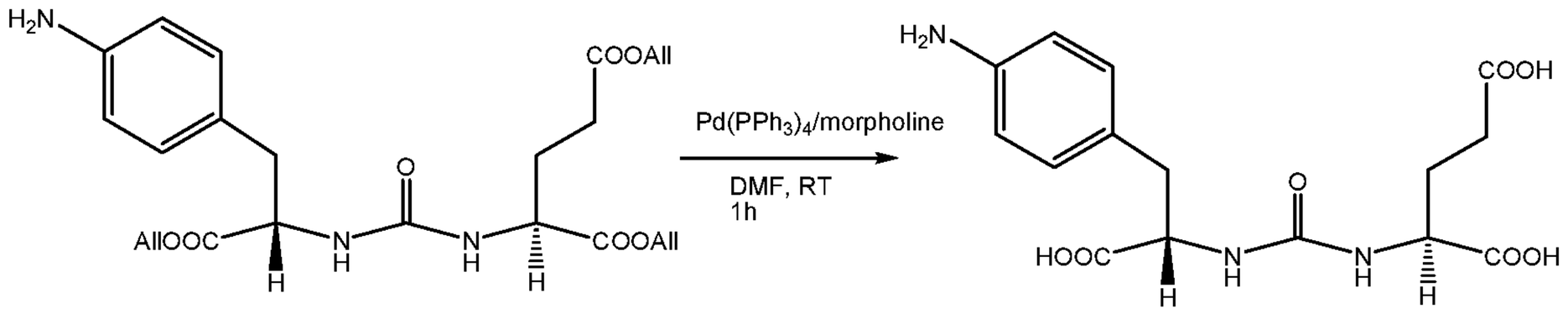


commercial available

To a stirred solution of diallyl glutamate (110 mg, 0.42 mmol) and triphosgene (43 mg, 0.14 mmol) in CH_2Cl_2 (4 mL) at -78 ºC was added Et_3N (180 µ L, 1.3 mmol) in CH_2Cl_2 (0.8 mL). The reaction mixture was allowed to warm to room temperature and stirred for 1.5 h. The phenylalanine derivative (140 mg, 0.251 mmol) in a solution of CH_2Cl_2 (1 mL) and Et_3N (70µ L, 0.5 mmol) was then added at -78 ºC and the reaction was stirred at room temperature for 12h. The solution was diluted with CH_2Cl_2, washed twice with H_2O, dried over $MgSO_4$ (anh.) and purified by column chromatography (Hexane:AcOEt 3:1) to give 100 mg (57%) (TLC in CH_2Cl_2:MeOH 20:1 Rf = 0.3, revealed with ninhydrin and uv light). ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 7.80-6.95 (13H, aromatics, N*H*Fmoc), 5.98-5.82 (m, 3H, 3-CH_2CHCH_2), 5.54 (bd, 1H, N*H*urea) ,5.43-5.19 (m, 7H, 3-CH_2CHCH_2, N*H*urea), 4.85-4.78 (m, 1H, C*H*(pheala.)), 4.67-4.50 (m, 9H, 3-C*H*$_2$CHCH$_2$, C*H*$_2$(Fmoc), C*H*(glu.)), 4.28 (t, 1H, C*H*(Fmoc)), 3.05 (d, 2H, C*H*$_2$(pheala.)), 2.53-2.33 (m, 2H, C*H*$_2$(glu.)), 2.25-2.11 and 1.98-1.80 (2m, 2H, C*H*$_2$(glu.)).



To a solution of the starting material (60 mg, 0.086 mmol) in CH_3CN (1 mL) was added Et_2NH (1 mL, 10 mmol). The reaction was stirred at room temperature for 40 min. The solvent was removed and the compound was purified by column chromatography (Hexane:AcOEt 2:1) to give 35 mg (85%). (TLC in CH_2Cl_2:MeOH 10:1 Rf = 0.5, started compound Rf = 0.75, revealed with ninhydrin (the compound has a violet color) and uv light).^{1}H-NMR ($CDCl_3$, 300 MHz) δ 6.85 and 6.55 (2d, 4H, aromatics), 5.98-5.82 (m, 3H, 3-CH_2CHCH_2), 5.56 (bd, 1H, N*H*urea) ,5.44-5.18 (m, 7H, 3-CH_2CHCH_2, N*H*urea), 4.79-4.72 (m,1H, C*H*(pheala.)), 4.65-4.49 (m, 7H, 3-CH_2CHCH_2, C*H*(glu.)), 3.64 (bs, 2H, NH_2), 3.02-2.89 (m,2H, CH_2(pheala.)), 2.49-2.31 (m, 2H, CH_2(glu.)), 2.20-2.09 and 1.91-1.78 (2m, 2H, CH_2(glu.)).

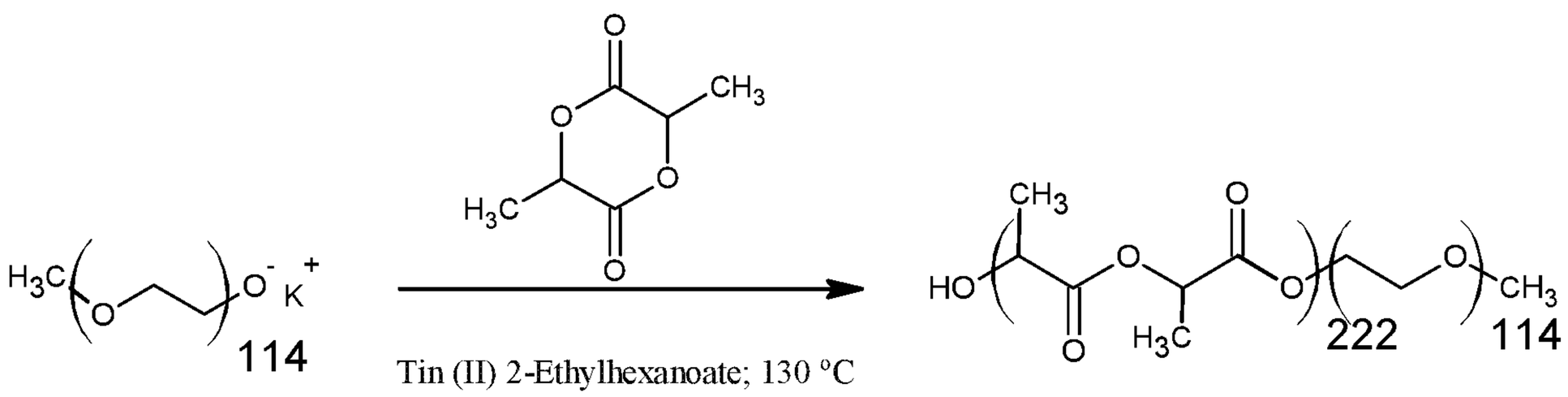


To a solution of the compound (50 mg, 0.105 mmol) in DMF (anh.; 1.5 mL) under argon was added $Pd(PPh_3)_4$ (21 mg, 0.018 mmol) and morpholine (154 µ L, 1.77 mmol) at 0 ºC. The reaction was stirred at room temperature for 1 h. The solvent was removed. The crude material was washed with CH_2Cl_2 twice, and dissolved in H_2O. To this solution was added a diluted solution of NaOH (0.01 N) until the pH was very basic. The solvent was removed under reduced pressure. The solid was washed again with CH_2Cl_2, AcOEt, and mixture of MeOH-CH_2Cl_2 (1:1), solved in H_2O and neutralized with Amberlite IR-120 H^+ resin. The solvent was evaporated and the compound was precipitated with MeOH, to give 25 mg (67 %) of GL1. ^{1}H-NMR (D_2O, 300 MHz) δ 7.08 and 6.79 (2d, 4H, aromatics), 4.21 (m, 1H, C*H*(pheala.)), 3.90 (m, 1H, C*H*(glu.)), 2.99 and 2.82 (2dd, 2H, CH_2(pheala.)), 2.22-2.11 (m, 2H, CH_2(glu.)), 2.05-1.70 (2m, 2H, CH_2(glu.)). ^{13}C-NMR (D_2O, 75 MHz) δ 176.8, 174.5, 173.9 (3 *C*OO). 153.3 (NH*C*ONH),

138.8 (H_2N-*C*(Ph)), 124.5, 122.9, 110.9 (aromatics), 51.3 (*C*H(pheala.)), 49.8 (*C*H(glu.)), 31.8 (*C*H_2(pheala.)), 28.4 and 23.6 (2*C*H_2-glu.)). Mass ESI: 354.19 [M + H^+], 376.23 [M + Na^+].

EXAMPLE 3: Preparation of PLA-PEG

The synthesis is accomplished by ring opening polymerization of d,l-lactide with α-hydroxy- ω -methoxypoly(ethylene glycol) as the macro-initiator, and performed at an elevated temperature using Tin (II) 2-Ethyl hexanoate as a catalyst, as shown below(PEG Mn ≈ 5,000 Da; PLA Mn ≈ 16,000 Da; PEG-PLA Mn ≈ 21,000 Da)



The polymer is purified by dissolving the polymer in dichloromethane, and precipitating it in a mixture of hexane and diethyl ether. The polymer recovered from this step shall be dried in an oven.

EXAMPLE 4: PLA-PEG-Ligand Preparation

The synthesis starts with the conversion of FMOC, BOC lysine to FMOC, BOC, Allyl lysine by reacting the FMOC, BOC lysine with allyl bromide and potassium carbonate in dimethyl formamide, followed by treatment with diethyl amine in acetonitrile. The BOC, Allyl lysine is then reacted with triphosgene and diallyl glutamate, followed by treatment with trifluoracetic acid in methylene chloride to form the compound “GL2P”.

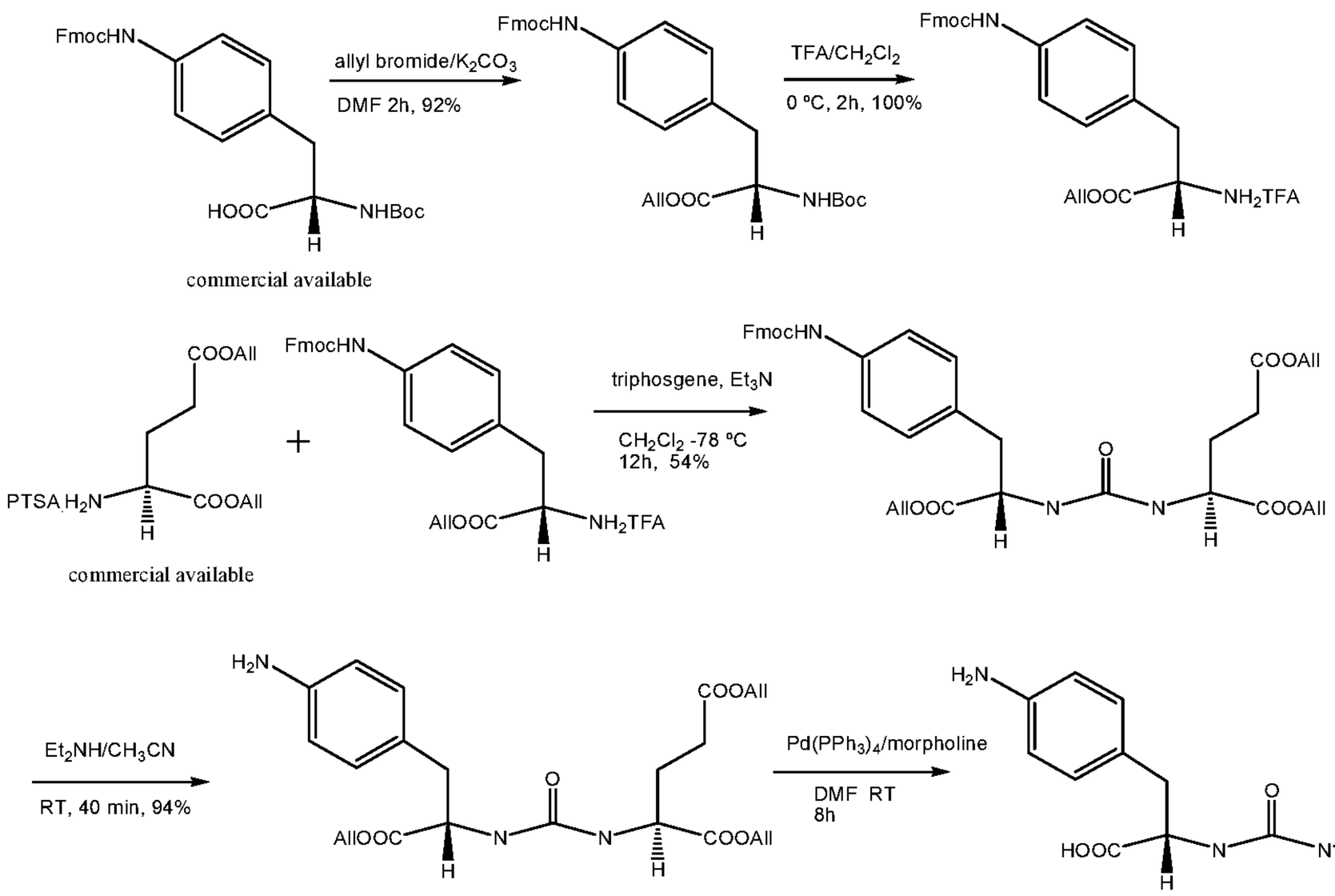
The side chain amine of lysine in the GL2P is then pegylated by the addition of Hydroxyl-PEG-Carboxlyic acid with EDC and NHS. The conjugation of GL2P to PEG is via an amide linkage. The structure of this resulting compound is labeled “HO-PEG-GL2P”. Following the pegylation, ring opening polymerization (ROP) of d,l-lactide with the hydroxyl group in the HO-PEG-GL2P as initiator is used to attach a polylactide

block polymer to HO-PEG-GL2P via an ester bond yielding "PLA-PEG-GL2P". Tin (II) 2-Ethyl hexanoate is used as a catalyst for the ring opening polymerization.

Lastly, the allyl groups on the PLA-PEG-GL2P are removed using morpholine and tetrakis(triphenylphosphine) palladium (as catalyst) in dichloromethane, to yield the final product PLA-PEG-Ligand. The final compound is purified by precipitation in 30/70% (v/v) diethyl ether/hexane.

EXAMPLE 5: Nanoparticle Preparation- Nanoprecipitation

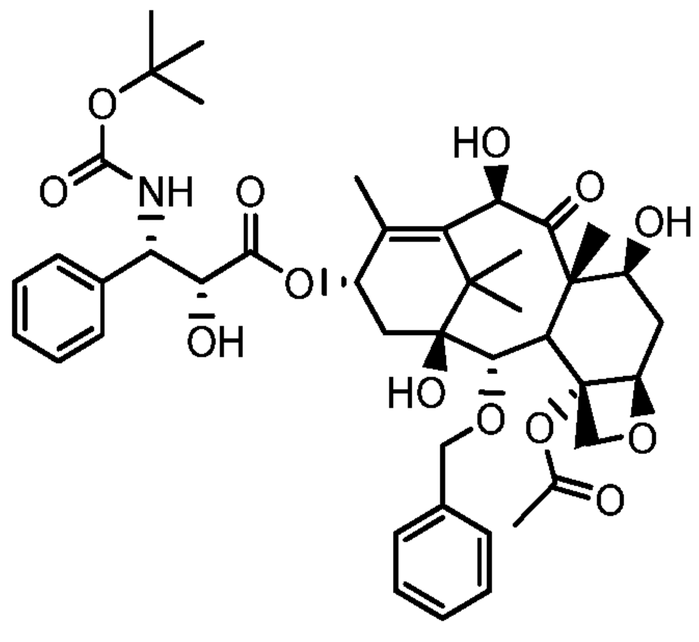
Nanoparticles can be prepared using GL1, GL2 or any desired ligand. The urea-based PSMA inhibitor GL2, which has a free amino group located in a region not critical for PSMA binding, is synthesized from commercially available starting materials Boc-Phe(4NHFmoc)-OH and diallyl glutamic acid in accordance with the procedure shown in Scheme 1. Nanoparticles are formed using nanoprecipitation: The polymer ligand conjugate is dissolved in a water miscible organic solvent together with a drug other agent for tracking particle uptake. Additional non-functionalized polymer can be included to modulate the ligand surface density. The polymer solution is dispersed in an aqueous phase and the resulting particles are collected by filtration. The particles can be dried or immediately tested for cell uptake in vitro or anti-prostate tumor activity in vivo.



SCHEME 1

EXAMPLE 6: Nanoparticle Preparation – Emulsion Process

An organic phase is formed composed of 5% solids (wt%) including 2% poly(lactide-co-glycolide)-poly(ethylene glycol) diblock copolymer (PLGA-PEG; 45kDa-5kDa), 2% poly(D,L-lactide) (PLA; 8.5kDa), and 1% docetaxel (DTXL) wherein docetaxel has the following structure:



The organic solvents are ethyl acetate (EA) and benzyl alcohol (BA) where BA comprises 20% (wt%) of the organic phase. BA is used in part to solublize the docetaxel. The organic phase is mixed with an aqueous phase at approximately a 1:5 ratio (oil phase:aqueous phase) where the aqueous phase is composed of 0.5% sodium cholate, 2% BA, and 4% EA (wt%) in water. The primary emulsion is formed by the combination of the two phases under simple mixing or through the use of a rotor stator homogenizer. The primary emulsion is then formed into a fine emulsion through the use of a probe sonicator or a high pressure homogenizer.

The fine emulsion is then quenched by addition to a chilled quench (0-5ºC) of deionized water under mixing. The quench:emulsion ratio is approximately 8.5:1. Then a solution of 25% (wt%) of Tween 80 is added to the quench to achieve approximately 2% Tween 80 overall. The nanoparticles are then isolated through either centrifugation or ultrafiltration/diafiltration. The nanoparticle suspension may then be frozen with a cyroprotectant, such as 10 wt% sucrose.

EXAMPLE 7: PSMA Targeted Docetaxel Nanoparticle Preparation – Emulsion Process

Prostate-Specific Membrane Antigen (PSMA) targeted docetaxel nanoparticles were prepared via an emulsion process for use in connection with the study described in Example 8 below. In a first step, an organic phase comprising 30% solids (wt%) was formed by mixing 2.34 kg (23.4%) PLA-PEG (16kDa-5kDa) of Example 3 with 0.06kg (0.6%) PLA-PEG-GL2 (16kDa-5kDa) of Example 4, and 6% docetaxel in the presence of organic solvents (5.53 kg of ethyl acetate and 1.47 kg of benzyl alcohol). Benzyl alcohol was used in part to solubilize the docetaxel.

In a next step, the organic phase was mixed with an aqueous phase at approximately a 1:2 weight ratio (organic phase:aqueous phase). The aqueous phase was formed by mixing 0.2kg of sodium cholate, 0.4kg of benzyl alcohol, and 0.8kg of ethyl acetate (wt%) in water (18.6kg).

The primary emulsion was formed by the combination of the two phases utilizing an overhead batch high-shear mixer. The primary emulsion was formed into a fine emulsion through the use of a high pressure homogenizer.

The fine emulsion was then quenched by addition to chilled (0-5ºC) deionized water under mixing. The quench:emulsion ratio was approximately 10:1. Then a 35% Tween 80 (15kg) solution in water (wt%) was added to the quench to dissolve any unencapsulated docetaxel.

The resulting nanoparticles were isolated and concentrated through ultrafiltration/diafiltration. Sucrose and hydroxypropyl-β-cyclodextrin were added to the nanoparticle suspension to serve as a cryoprotectants/lyoprotectants, at an amount resulting in a final suspension containing 5 wt% sucrose and 7.5% hydroxypropyl-β-cyclodextrin. The nanoparticle suspension was passed through a filtration train terminating in a 0.2 micron sterilizing grade filter. The nanoparticle suspension was filled into glass vials, lyophilized, stoppered, and sealed through capping.

EXAMPLE 8: In-Vivo docetaxel nanoparticle study

In vivo mouse syngeneic xenograft studies were performed to evaluate combination activity of the checkpoint inhibitor, anti-PD-1, and the PSMA targeted docetaxel polymeric nanoparticles of Example 7. Studies were run in 6 to 8 week old female BALB/c mice bearing subcutaneous mouse colon CT-26 tumors.

When tumors reached approximately 100mm^3 in size, mice were treated with either:

i) isotype control (clone 2A3, 10mg/kg i.p.);

ii) mouse anti-PD-1 (clone RMP1-14, 10mg/kg i.p.);

iii) docetaxel nanoparticle (Example 7 10mg/kg i.v.),;

iv) docetaxel (Taxotere, 2.5mg/kg i.v.);

v) docetaxel nanoparticle (Example 7) in combination with anti-PD-1 dosed simultaneously on an every 4 day schedule (q4d) for a total of 5 doses; or

vi) docetaxel with anti-PD-1 dosed simultaneously on an every 4 day schedule (q4d) for a total of 5 doses.

Data was graphed as mean and SEM with 10 mice per treatment group. The data showed that there was no combination activity when docetaxel (Taxotere) was tested at a well-tolerated dose in combination with anti-PD-1 (Figure 9). However, studies performed showed that combining anti-PD-1 with docetaxel nanoparticles enhanced anti-tumor response in the CT26 mouse xenograft model which resulted in a day 22 tumor growth inhibition (TGI) of 88% and 2 complete regressions compared to 47 to 66% tumor growth inhibition with single agent treatment (Figure 3).

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

INCORPORATION BY REFERENCE

The entire contents of all patents, published patent applications, websites, and other references cited herein are hereby expressly incorporated herein in their entireties by reference.

What is claimed:

1. A therapeutic composition comprising:

a therapeutic nanoparticle comprising a therapeutic agent and a diblock poly(lactic) acid-poly(ethylene)glycol copolymer, wherein the therapeutic nanoparticle comprises about 10 to about 30 weight percent poly(ethylene)glycol; and

an immune checkpoint inhibitor, wherein the immune checkpoint inhibitor is an anti-PD- 1 antibody.

2. The therapeutic composition of claim 1, wherein the anti-PD-1 antibody is encapsulated in the therapeutic nanoparticle with the therapeutic agent.

3. The therapeutic composition of claim 1, wherein the anti-PD antibody is conjugated to the surface of the nanoparticle.

4. The therapeutic composition of claim 1, wherein the therapeutic agent is a chemotherapeutic agent.

5. The therapeutic composition of claim 1, wherein the therapeutic nanoparticle comprises a targeting ligand.

6. The therapeutic composition of claim 5, wherein the targeting ligand is a prostate-specific membrane antigen (PSMA) targeting ligand.

7. The therapeutic composition of claim 1, wherein the poly(lactic) acid-poly(ethylene)glycol copolymer has a number average molecular weight of about 15kDa to about 30kDa poly(lactic acid) and a number average molecular weight of about 4kDa to about 6kDa poly(ethylene)glycol.

8. A method of treating a patient with cancer, the method comprising:

administering a) a therapeutic nanoparticle comprising a therapeutic agent and a diblock poly(lactic) acid-poly(ethylene)glycol copolymer, wherein the therapeutic nanoparticle comprises about 10 to about 30 weight percent poly(ethylene)glycol; and

b) an immune checkpoint inhibitor.

9. The method of claim 8, wherein the immune checkpoint inhibitor is an anti-PD-1 antibody

10. The method of any one of claims 8-9, wherein the therapeutic nanoparticle comprises a prostate-specific membrane antigen (PSMA) targeting ligand.

11. A therapeutic nanoparticle comprising:

a therapeutic agent;

about 0.2 to about 20 weight percent of an antibody; and

about 50 to about 99.75 weight percent of a diblock poly(lactic) acid-poly(ethylene)glycol copolymer, wherein the therapeutic nanoparticle comprises about 10 to about 30 weight percent poly(ethylene)glycol.

12 The therapeutic nanoparticle of claim 11, comprising about 2 to about 5 weight percent of the antibody.

13. The therapeutic nanoparticle of claim 11, wherein the antibody is a monocolonal antibody.

14. The therapeutic nanoparticle of claim 11, wherein the antibody is an anti-PD-1 antibody.

15. The therapeutic nanoparticle of claim 11, wherein the antibody is associated with a hydrophobic counter ion.

16. The therapeutic nanoparticle of claim 11, wherein the poly(lactic) acid-poly(ethylene)glycol copolymer has a number average molecular weight of about 15kDa to about 20kDa poly(lactic acid) and a number average molecular weight of about 4kDa to about 6kDa poly(ethylene)glycol.

17. A method of enhancing anti-tumor response in a patient in need thereof, comprising:

co-administering to a patient in need thereof a therapeutically effective amount of a therapeutic nanoparticle, and an immune checkpoint inhibitor.

18. The method of claim 17, wherein the therapeutic nanoparticle comprises:

a therapeutic agent and a diblock poly(lactic) acid-poly(ethylene)glycol copolymer, wherein the therapeutic nanoparticle comprises about 10 to about 30 weight percent poly(ethylene)glycol.

19. The method according to claim 17 or 18, wherein the immune checkpoint inhibitor is an Anti-PD-1 antibody.

20. A pharmaceutical composition comprising:

a therapeutic nanoparticle comprising a therapeutic agent and a diblock poly(lactic) acid-poly(ethylene)glycol copolymer, and a prostate-specific membrane antigen (PSMA) targeting ligand, wherein the therapeutic nanoparticle comprises about 10 to about 30 weight percent poly(ethylene)glycol, wherein said composition is lyophilized.

21. The composition of claim 20, wherein the composition further comprises an Anti-PD-1 antibody.

FIG. 1

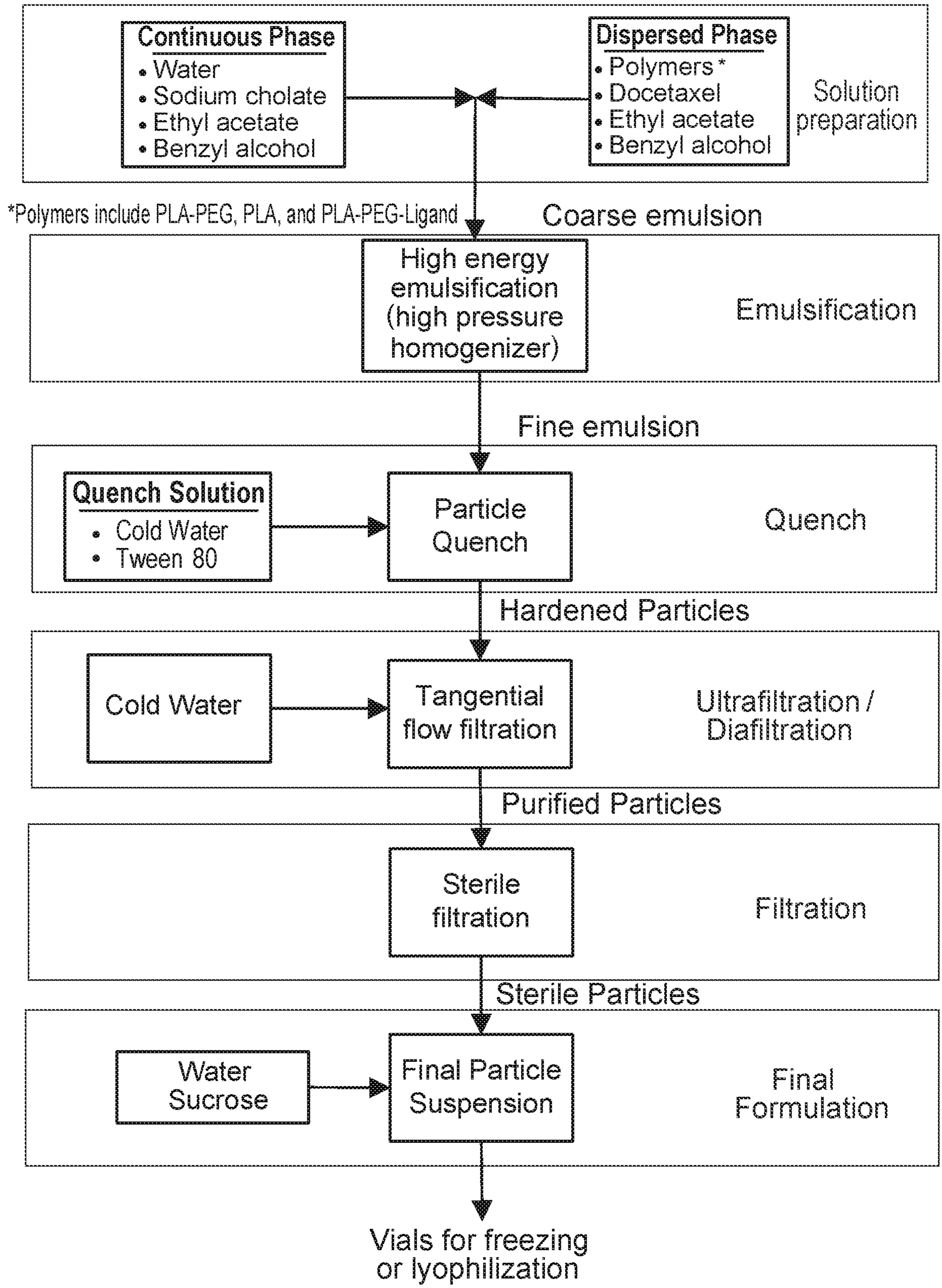

Continuous Phase
• Water
• Sodium cholate
• Ethyl acetate
• Benzyl alcohol
Dispersed Phase
• Polymers*
• Docetaxel
• Ethyl acetate
• Benzyl alcohol
Solution preparation
*Polymers include PLA-PEG, PLA, and PLA-PEG-Ligand
Coarse emulsion
High energy emulsification (high pressure homogenizer)
Emulsification
Fine emulsion
Quench Solution
• Cold Water
• Tween 80
Particle Quench
Quench
Hardened Particles
Cold Water
Tangential flow filtration
Ultrafiltration / Diafiltration
Purified Particles
Sterile filtration
Filtration
Sterile Particles
Water Sucrose
Final Particle Suspension
Final Formulation
Vials for freezing or lyophilization

FIG. 2A

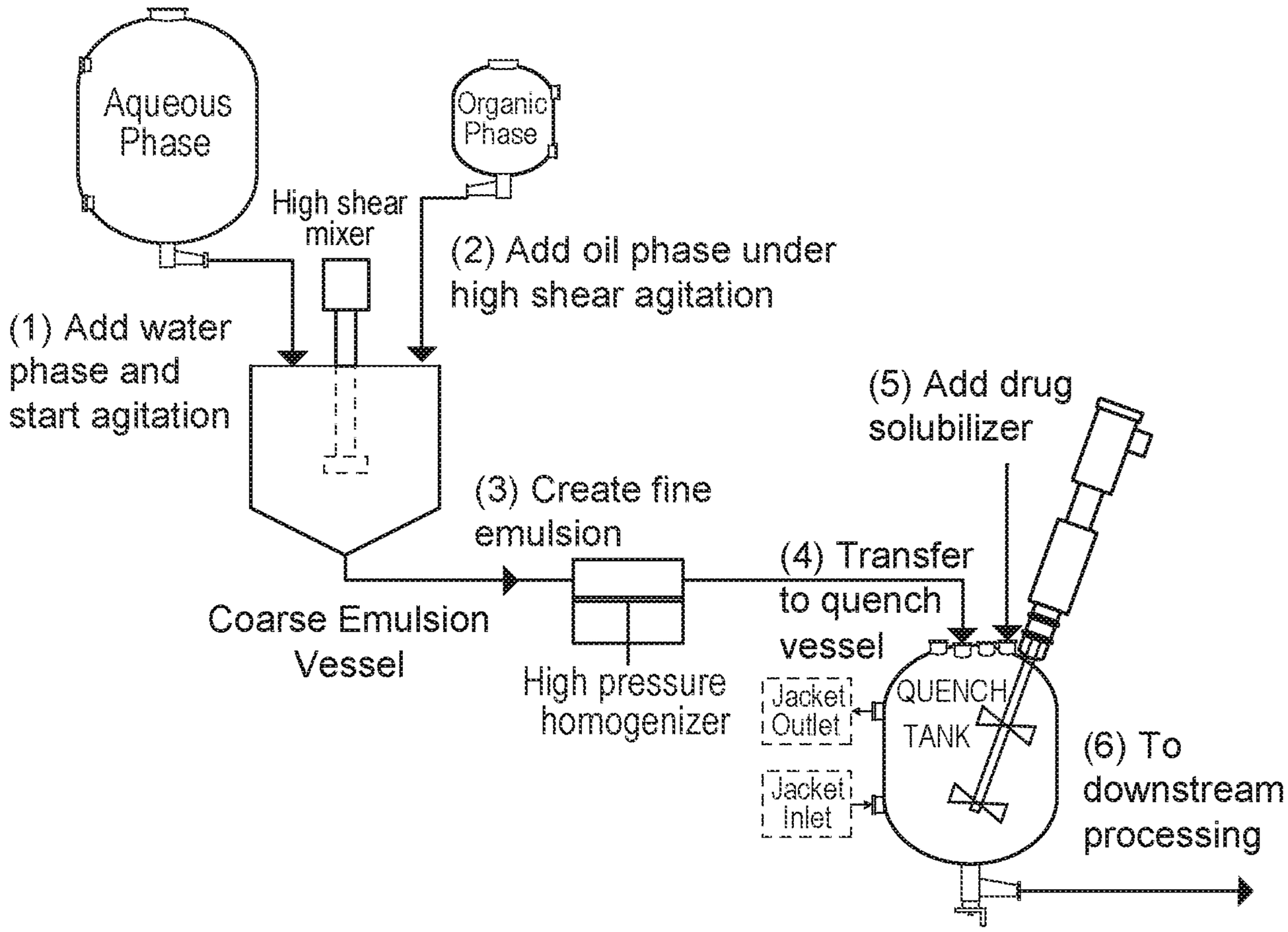

Aqueous Phase
Organic Phase
High shear mixer
(1) Add water phase and start agitation
(2) Add oil phase under high shear agitation
(3) Create fine emulsion
Coarse Emulsion Vessel
High pressure homogenizer
(4) Transfer to quench vessel
(5) Add drug solubilizer
QUENCH TANK
Jacket Outlet
Jacket Inlet
(6) To downstream processing

FIG. 2B

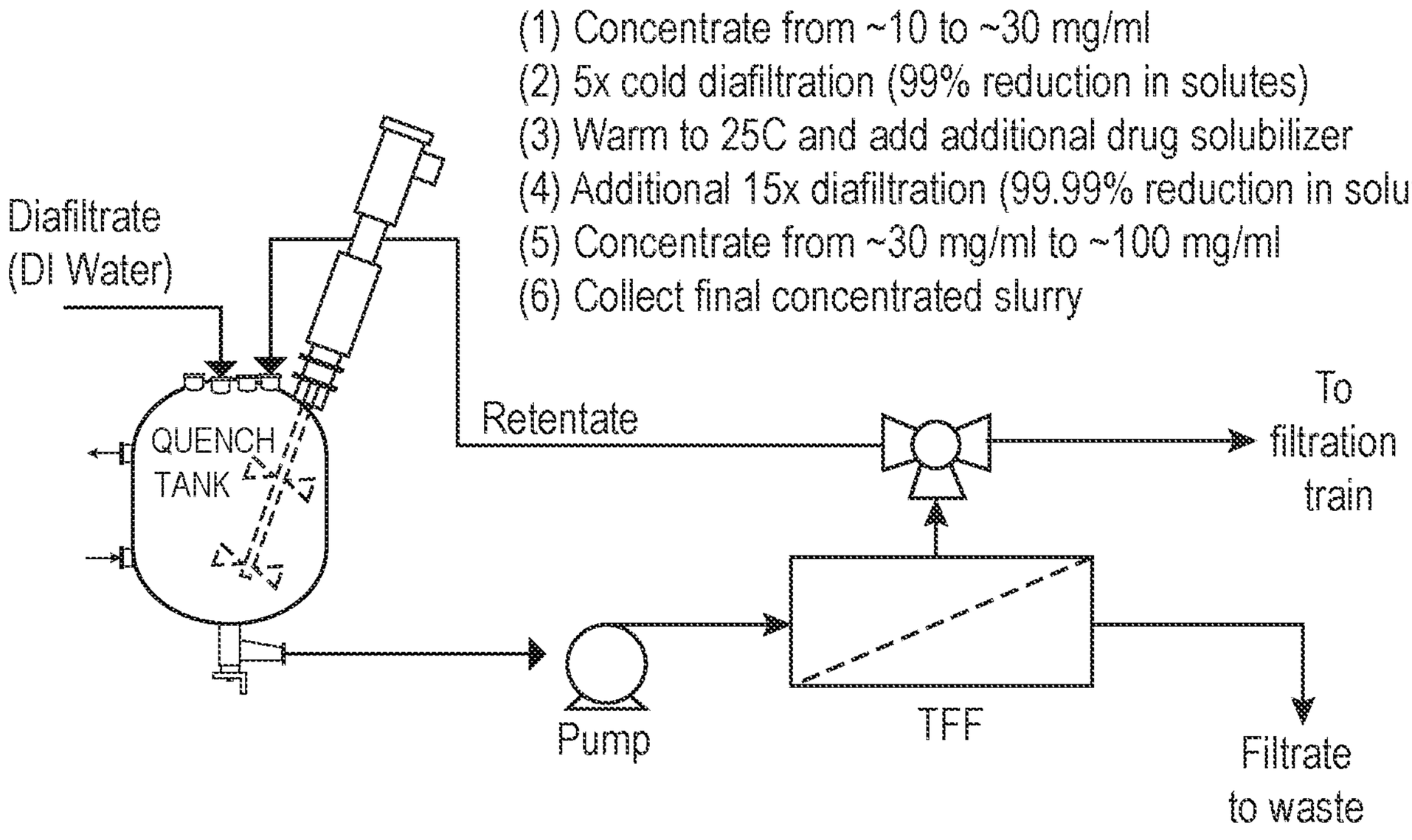

(1) Concentrate from ~10 to ~30 mg/ml
(2) 5x cold diafiltration (99% reduction in solutes)
(3) Warm to 25C and add additional drug solubilizer
(4) Additional 15x diafiltration (99.99% reduction in solutes)
(5) Concentrate from ~30 mg/ml to ~100 mg/ml
(6) Collect final concentrated slurry
Diafiltrate (DI Water)
QUENCH TANK
Retentate
To filtration train
Pump
TFF
Filtrate to waste

FIG. 3

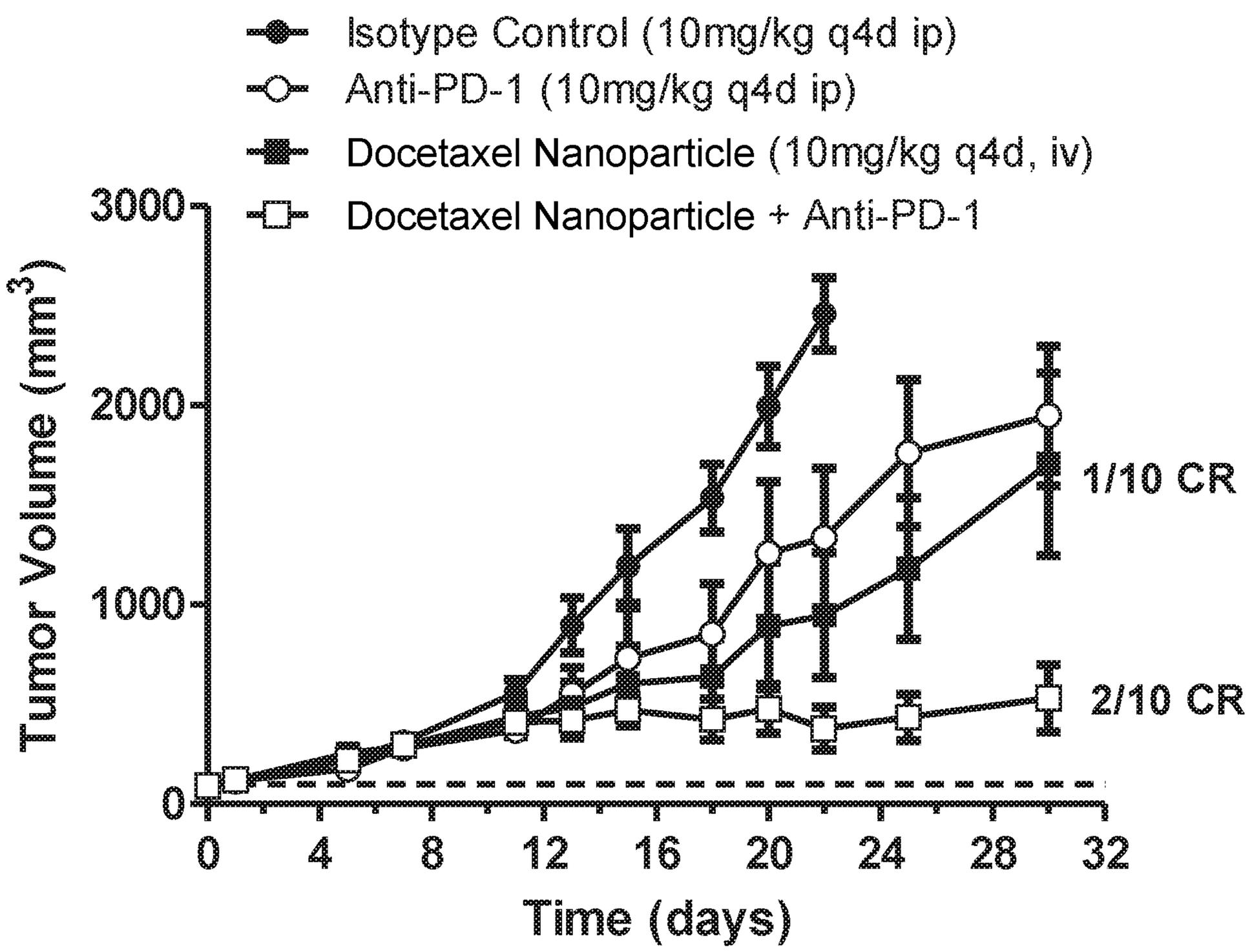

Isotype Control (10mg/kg q4d ip)
Anti-PD-1 (10mg/kg q4d ip)
Docetaxel Nanoparticle (10mg/kg q4d, iv)
Docetaxel Nanoparticle + Anti-PD-1
Tumor Volume (mm3)
3000
2000
1000
0
0
4
8
12
16
20
24
28
32
Time (days)
1/10 CR
2/10 CR

FIG. 4

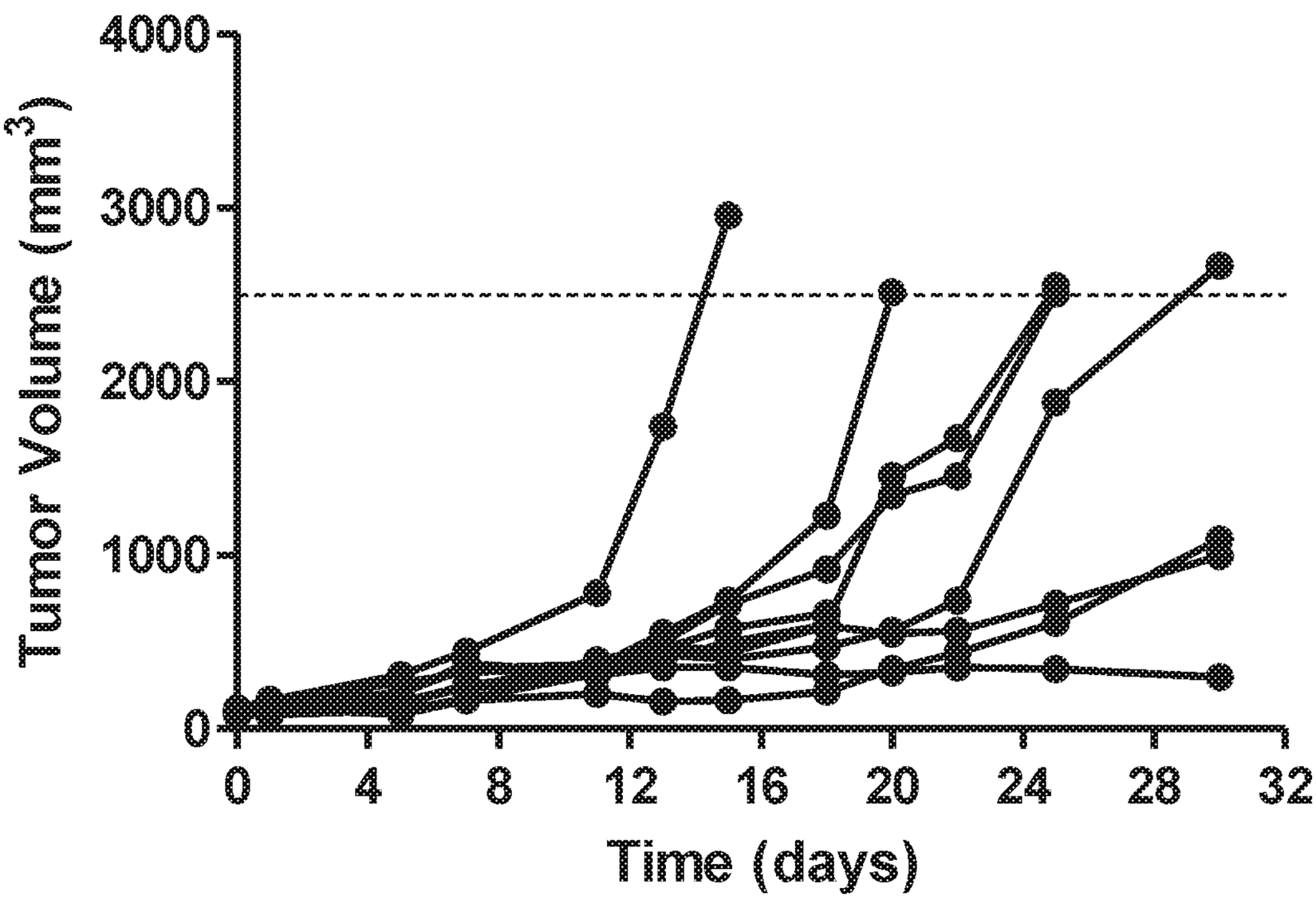

4000
3000
2000
1000
0
Tumor Volume (mm^3)
0
4
8
12
16
20
24
28
32
Time (days)

FIG. 5

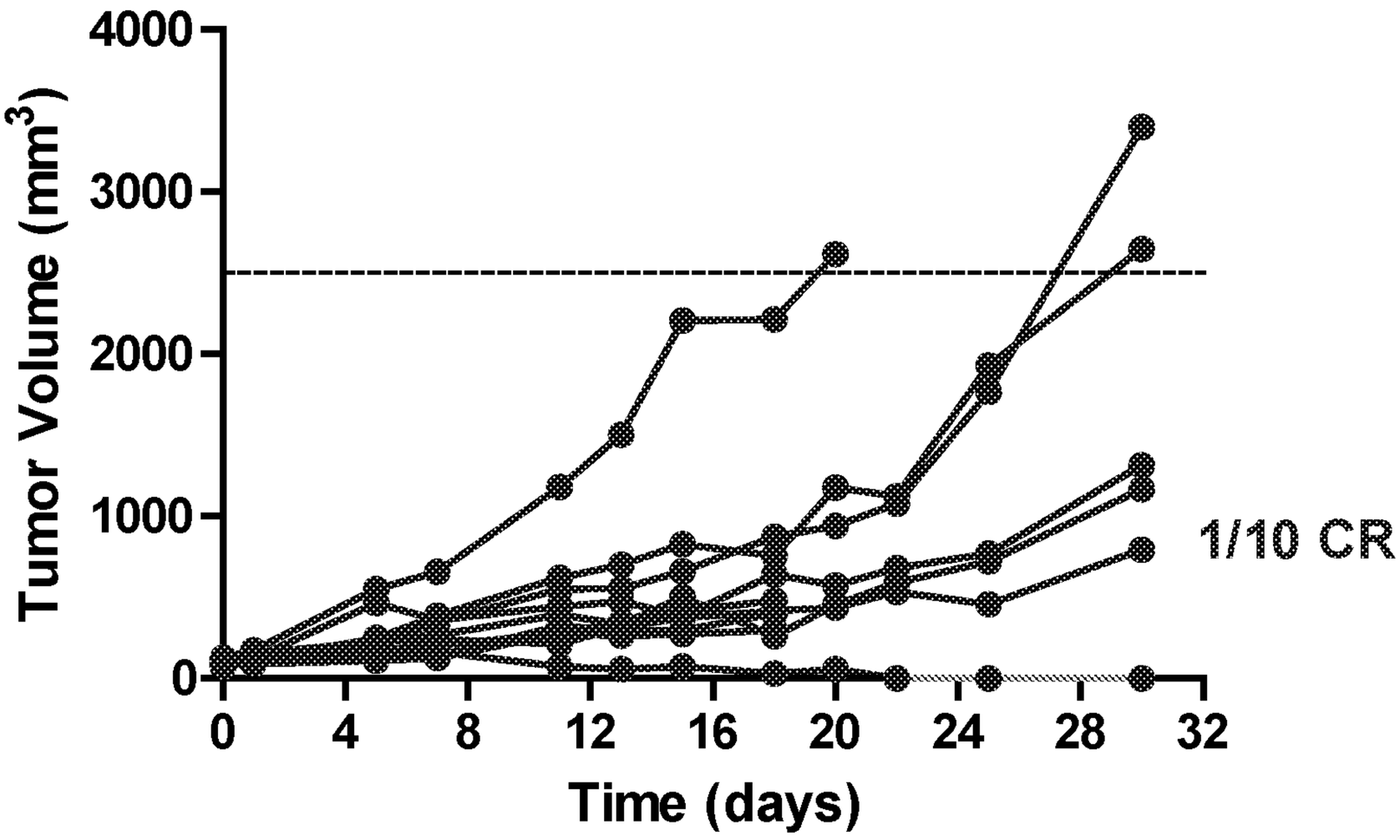

4000
3000
2000
1000
0
Tumor Volume (mm3)
0
4
8
12
16
20
24
28
32
Time (days)
1/10 CR

FIG. 6

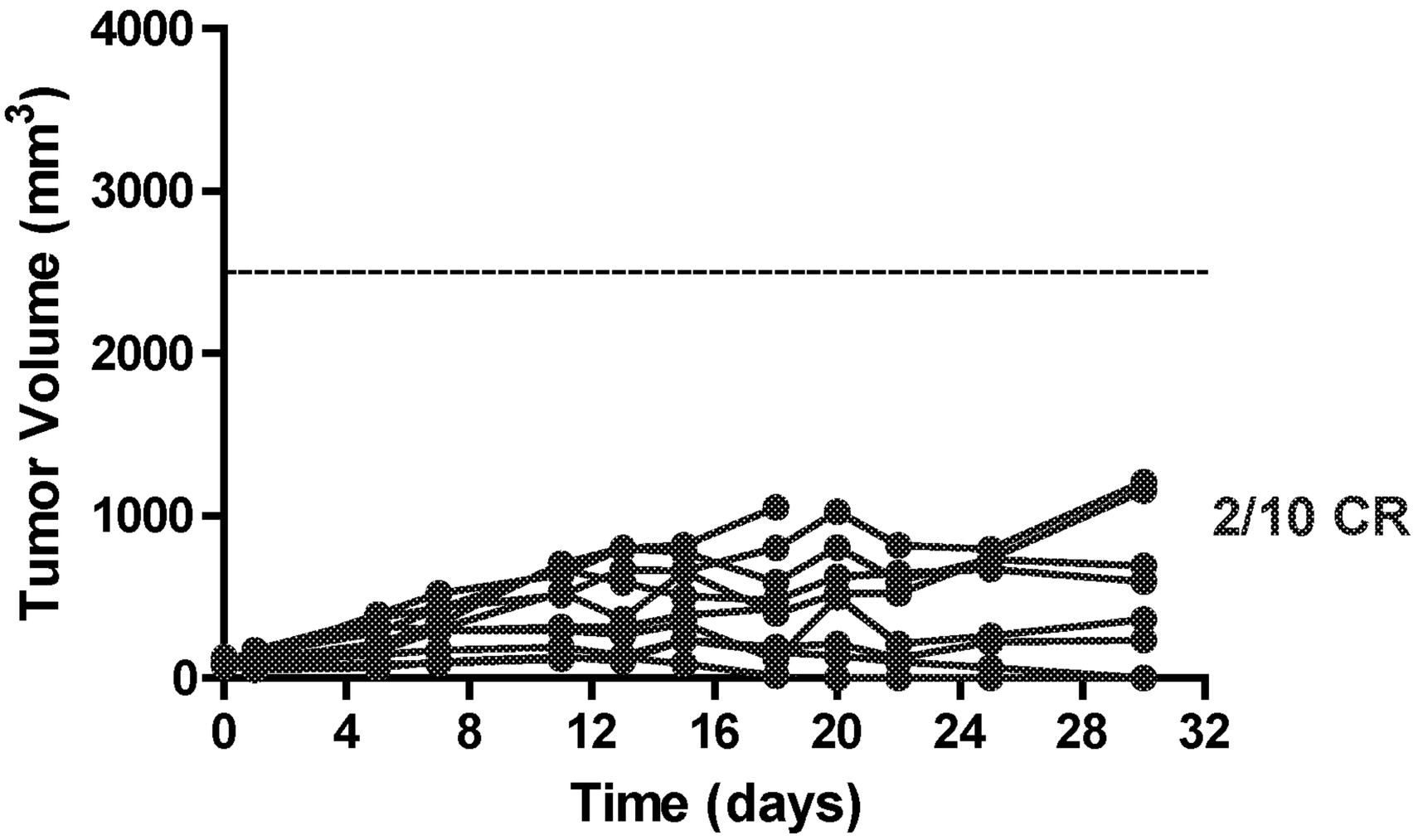

4000
3000
2000
1000
0
Tumor Volume (mm3)
0
4
8
12
16
20
24
28
32
Time (days)
2/10 CR

FIG. 7

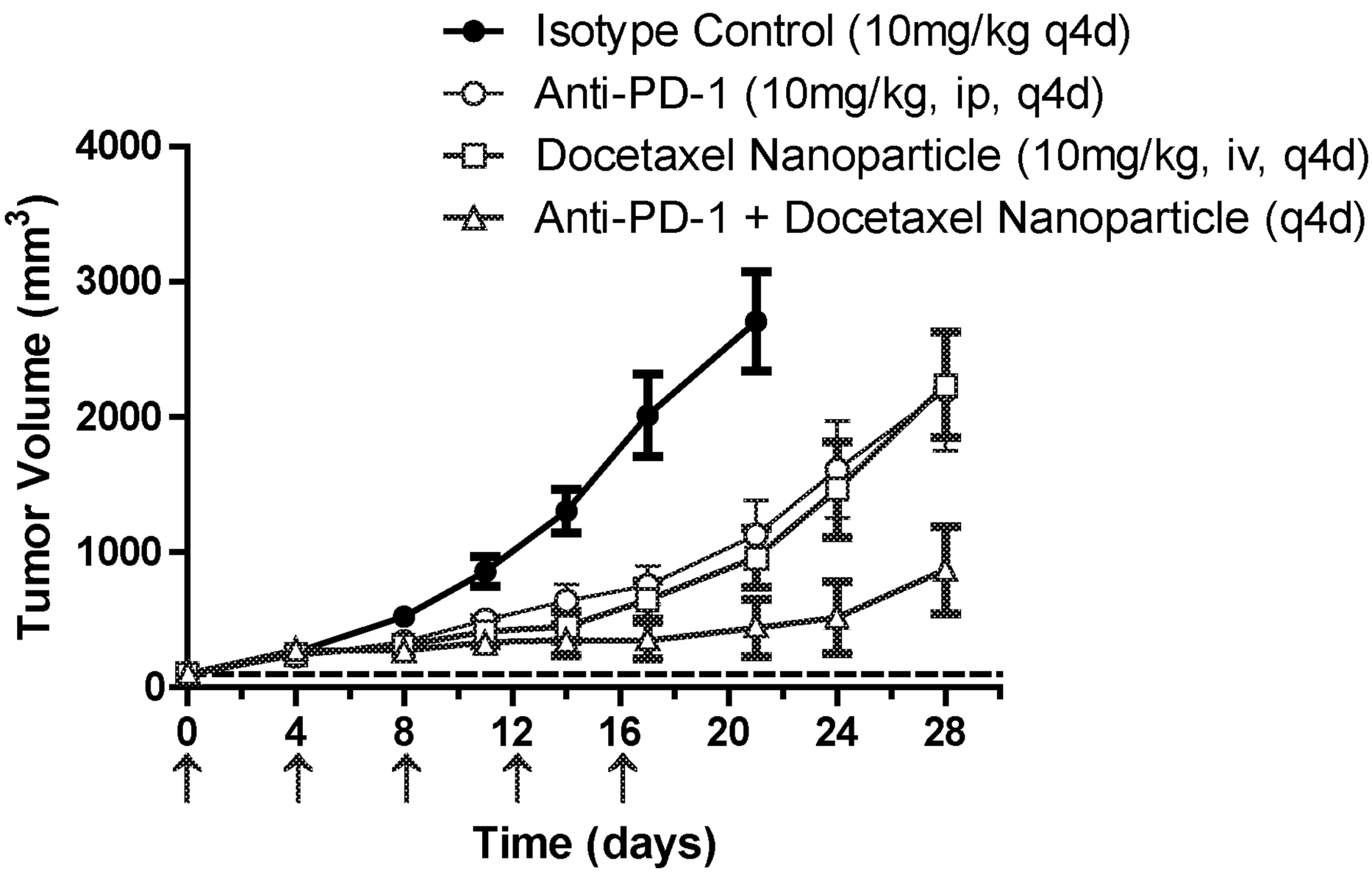

Isotype Control (10mg/kg q4d)
Anti-PD-1 (10mg/kg, ip, q4d)
Docetaxel Nanoparticle (10mg/kg, iv, q4d)
Anti-PD-1 + Docetaxel Nanoparticle (q4d)
Tumor Volume (mm3)
4000
3000
2000
1000
0
0
4
8
12
16
20
24
28
Time (days)

FIG. 8

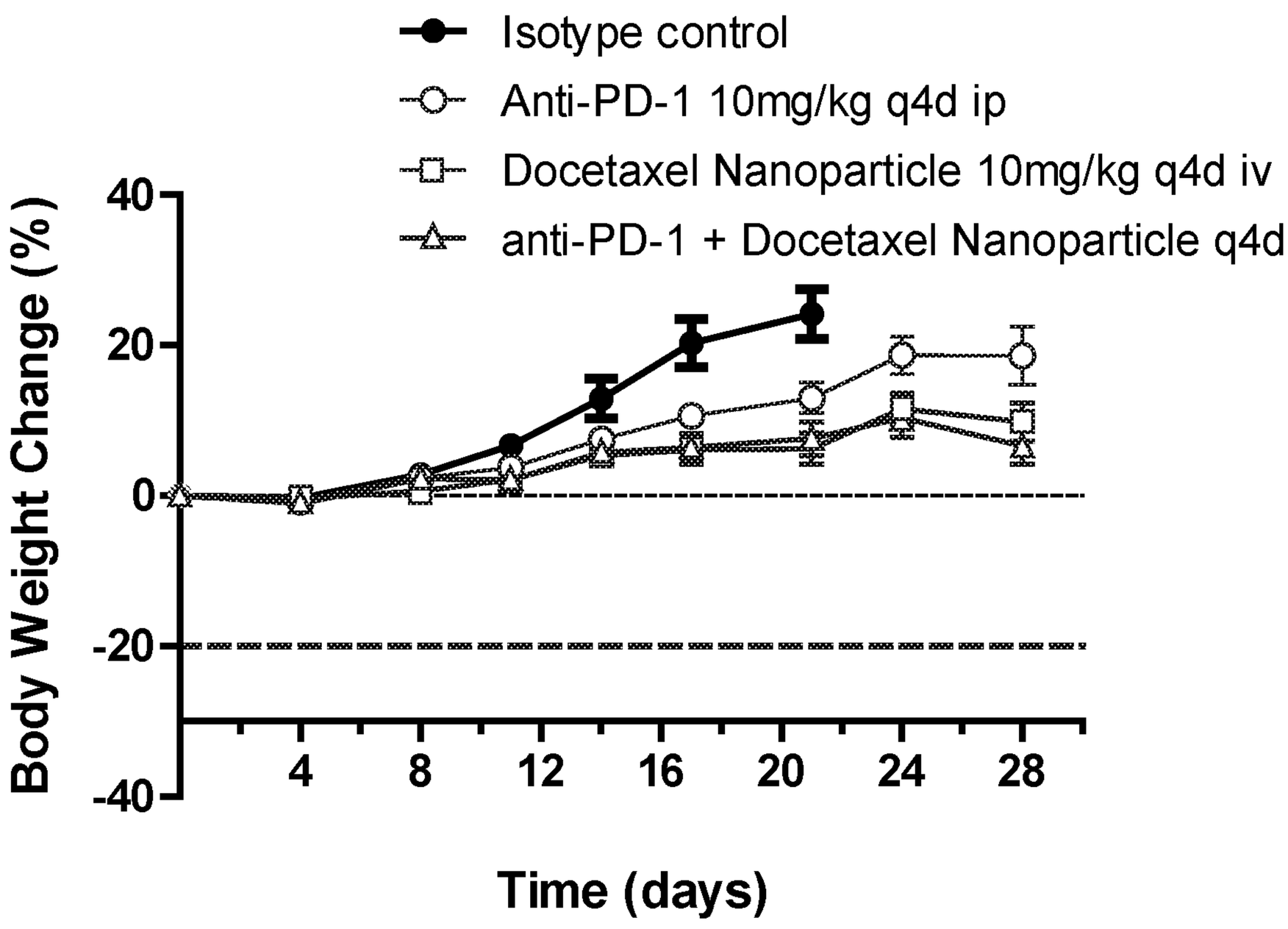

Isotype control
Anti-PD-1 10mg/kg q4d ip
Docetaxel Nanoparticle 10mg/kg q4d iv
anti-PD-1 + Docetaxel Nanoparticle q4d
Body Weight Change (%)
40
20
0
-20
-40
4
8
12
16
20
24
28
Time (days)

FIG. 9

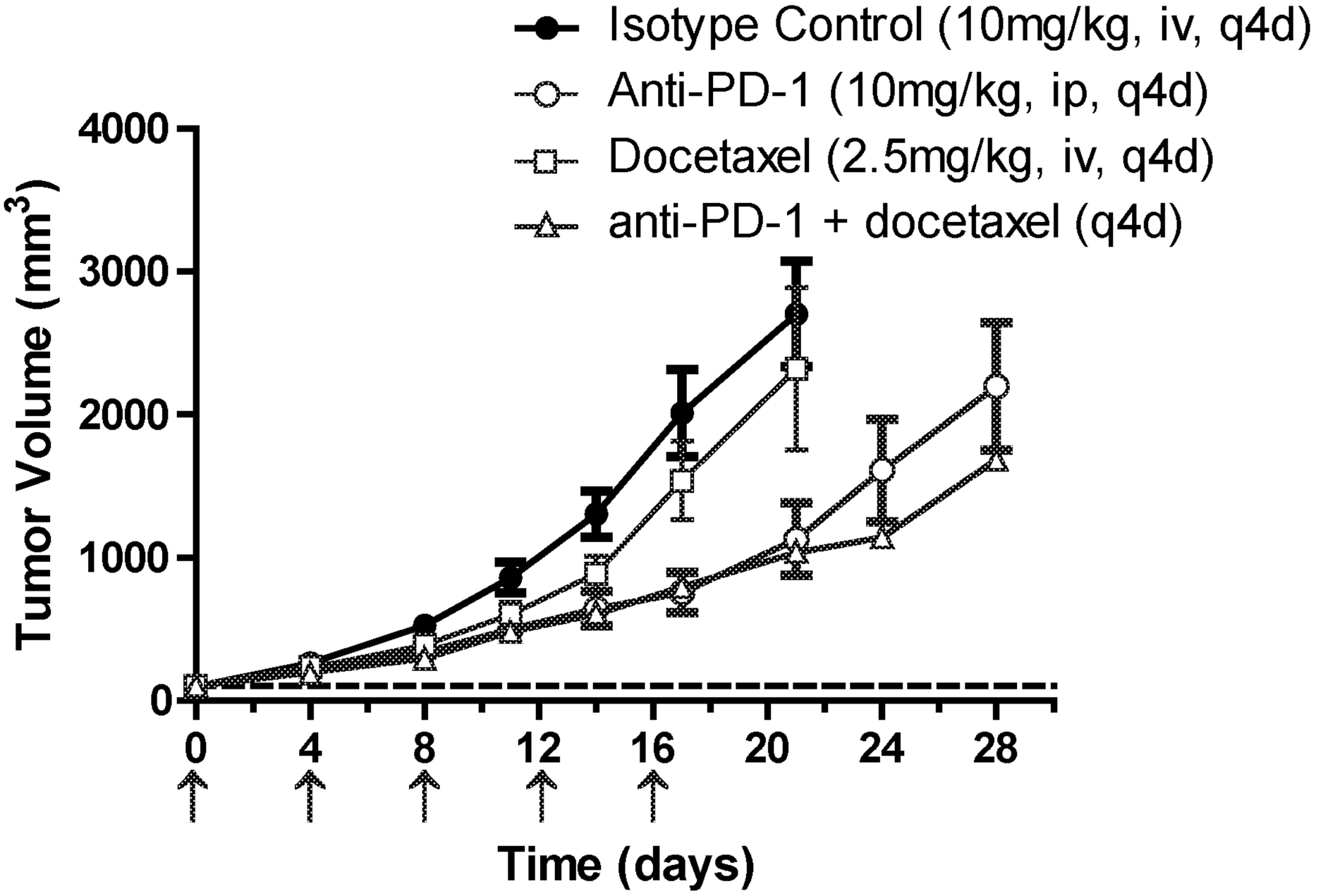

Isotype Control (10mg/kg, iv, q4d)
Anti-PD-1 (10mg/kg, ip, q4d)
Docetaxel (2.5mg/kg, iv, q4d)
anti-PD-1 + docetaxel (q4d)
Tumor Volume (mm3)
4000
3000
2000
1000
0
0
4
8
12
16
20
24
28
Time (days)

FIG. 10

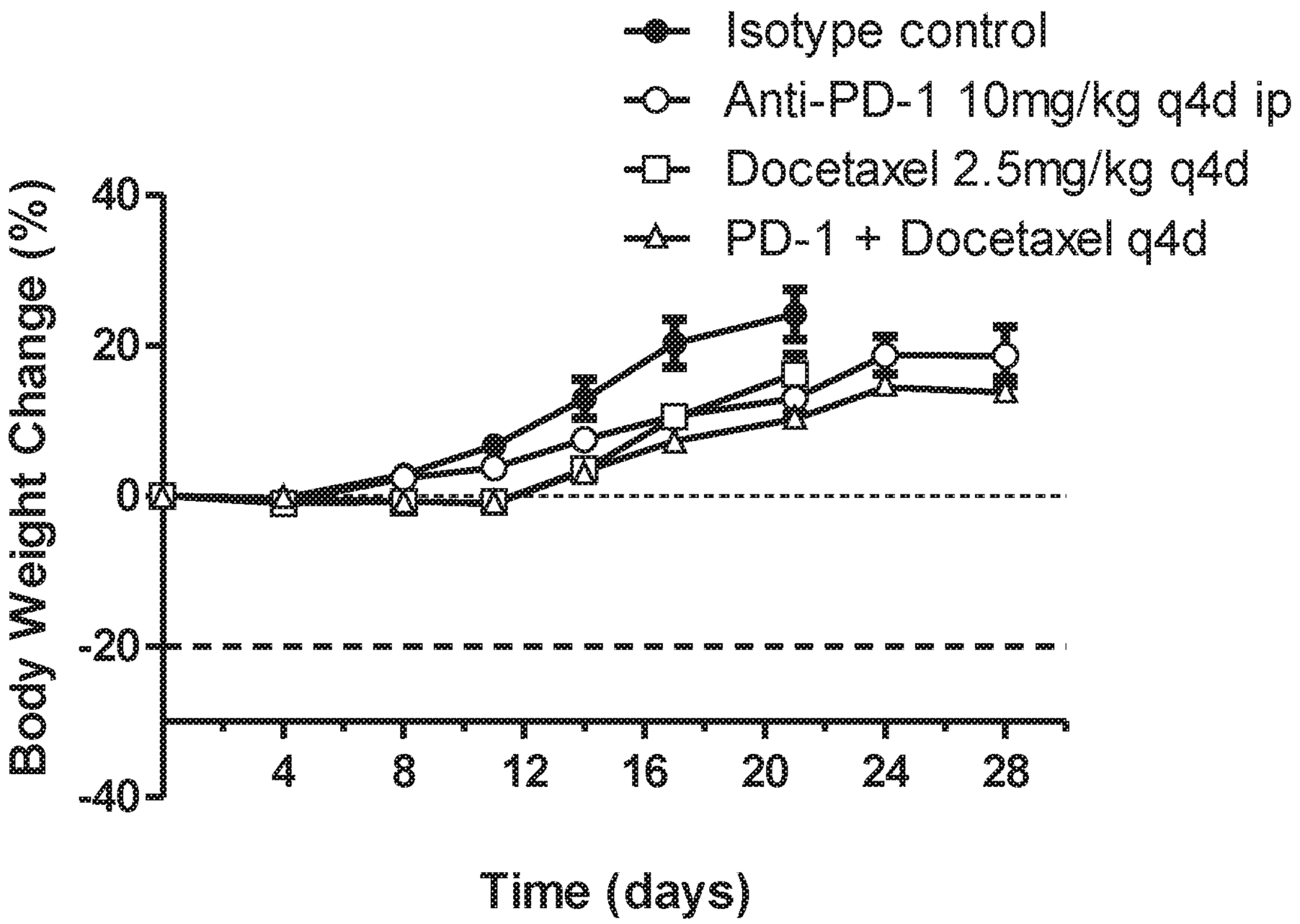

Isotype control
Anti-PD-1 10mg/kg q4d ip
Docetaxel 2.5mg/kg q4d
PD-1 + Docetaxel q4d
Body Weight Change (%)
40
20
0
-20
-40
4
8
12
16
20
24
28
Time (days)

FIG. 11

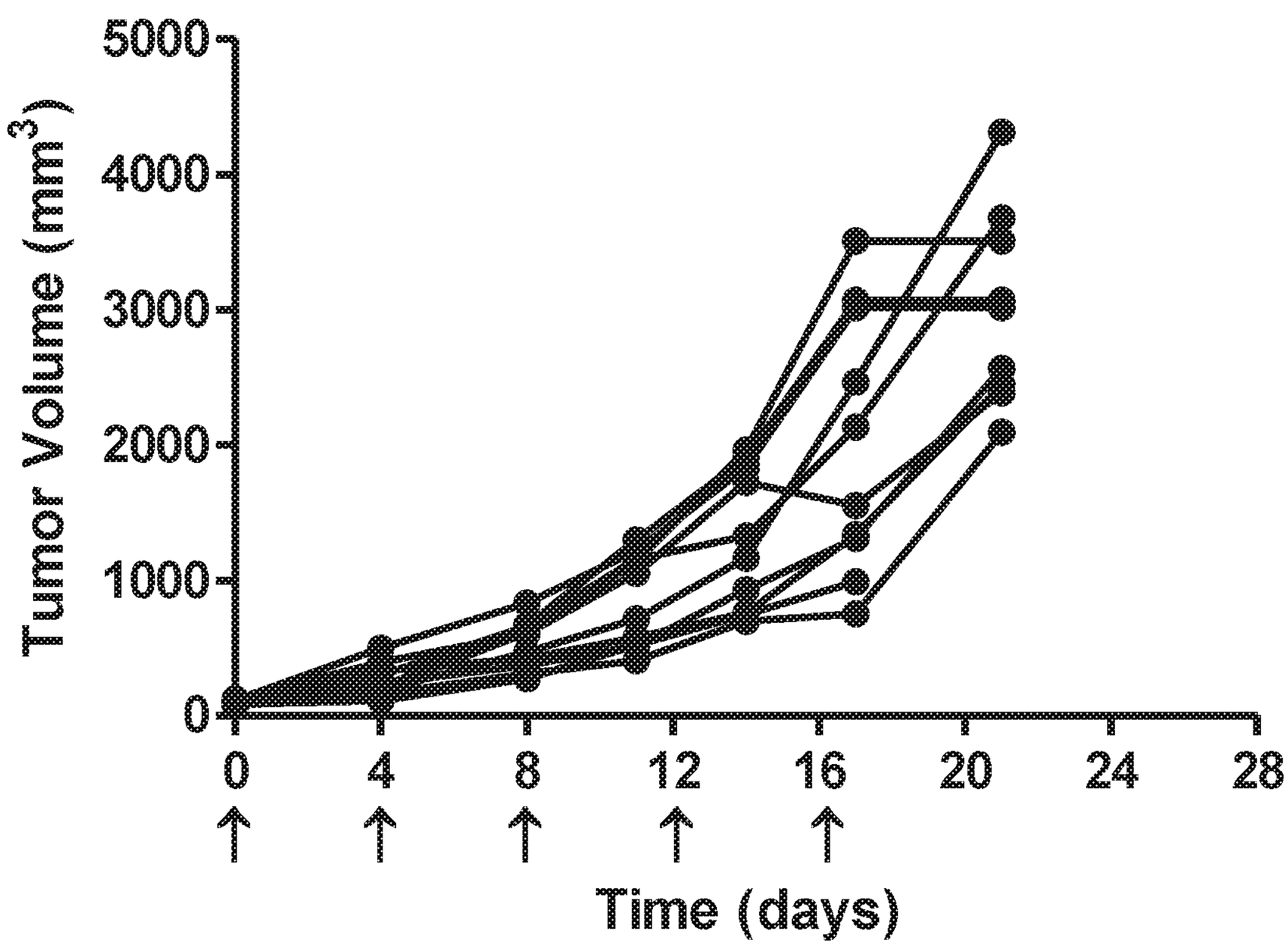

5000
4000
3000
2000
1000
0
Tumor Volume (mm^3)
0
4
8
12
16
20
24
28
Time (days)

FIG. 12

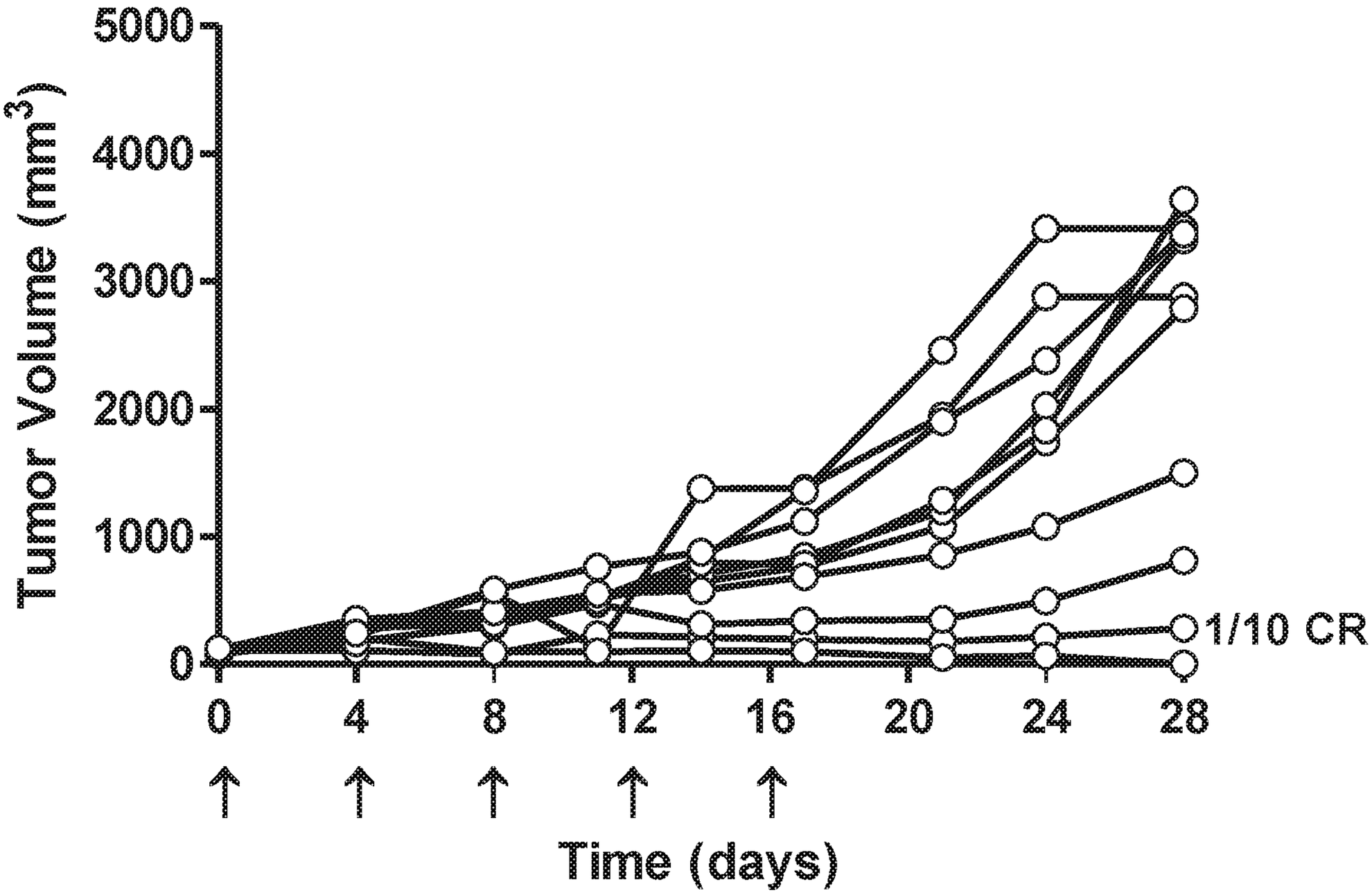

Tumor Volume (mm^3)
5000
4000
3000
2000
1000
0
1/10 CR
0
4
8
12
16
20
24
28
Time (days)

FIG. 13

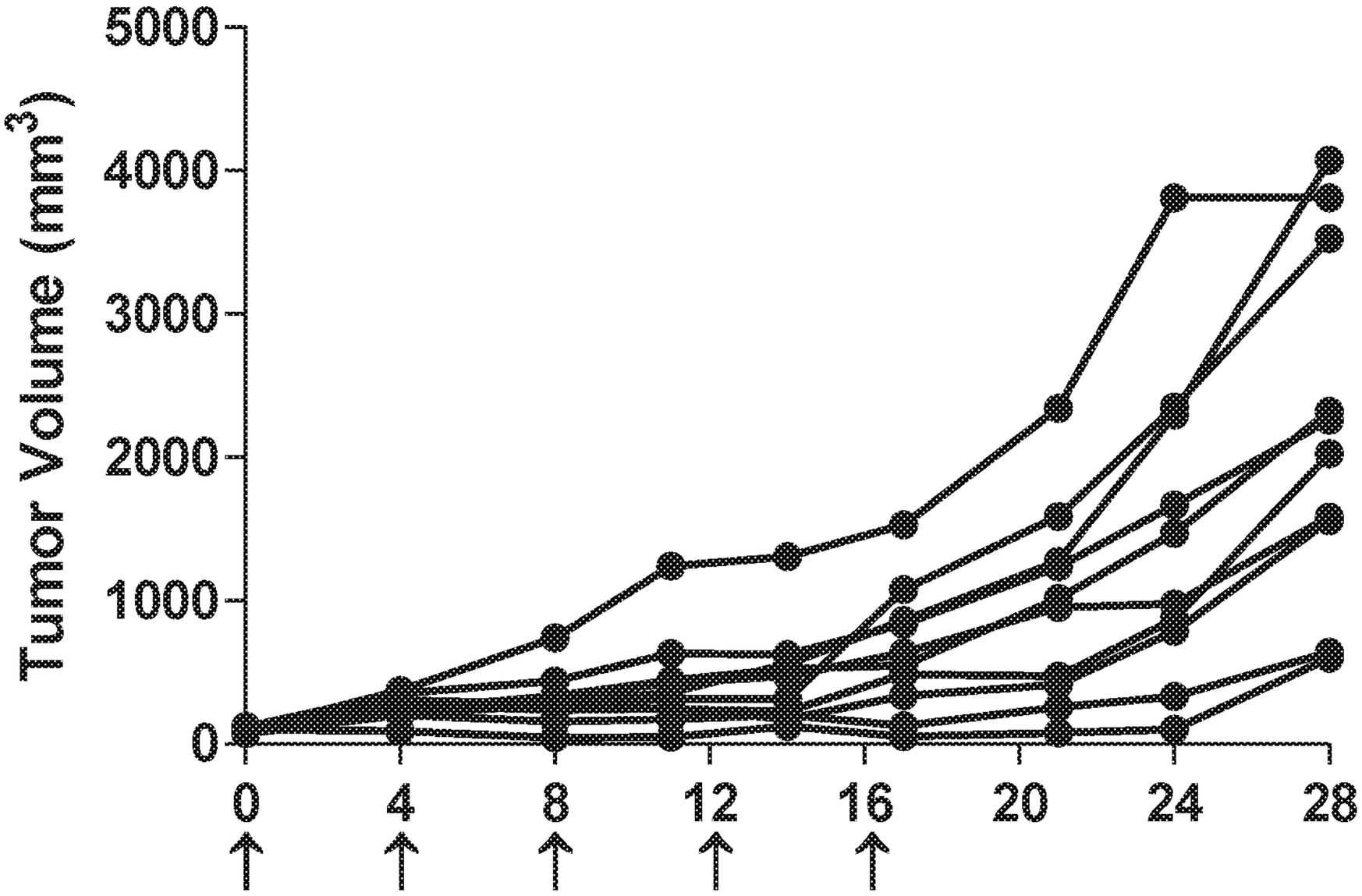

5000
4000
3000
2000
1000
0
Tumor Volume (mm3)
0
4
8
12
16
20
24
28
Time (days)

FIG. 14

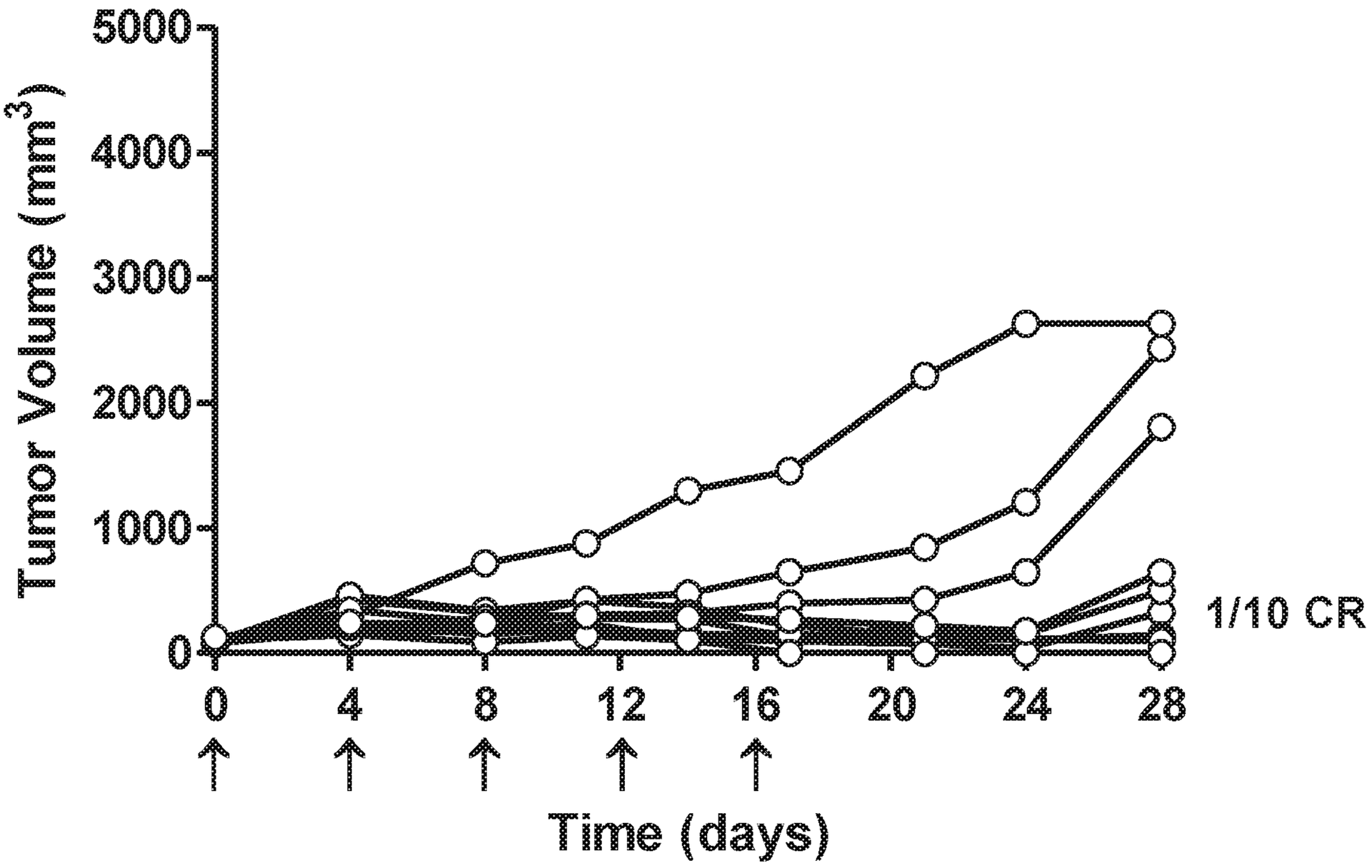

Tumor Volume (mm^3)
5000
4000
3000
2000
1000
0
1/10 CR
0
4
8
12
16
20
24
28
Time (days)

FIG. 15

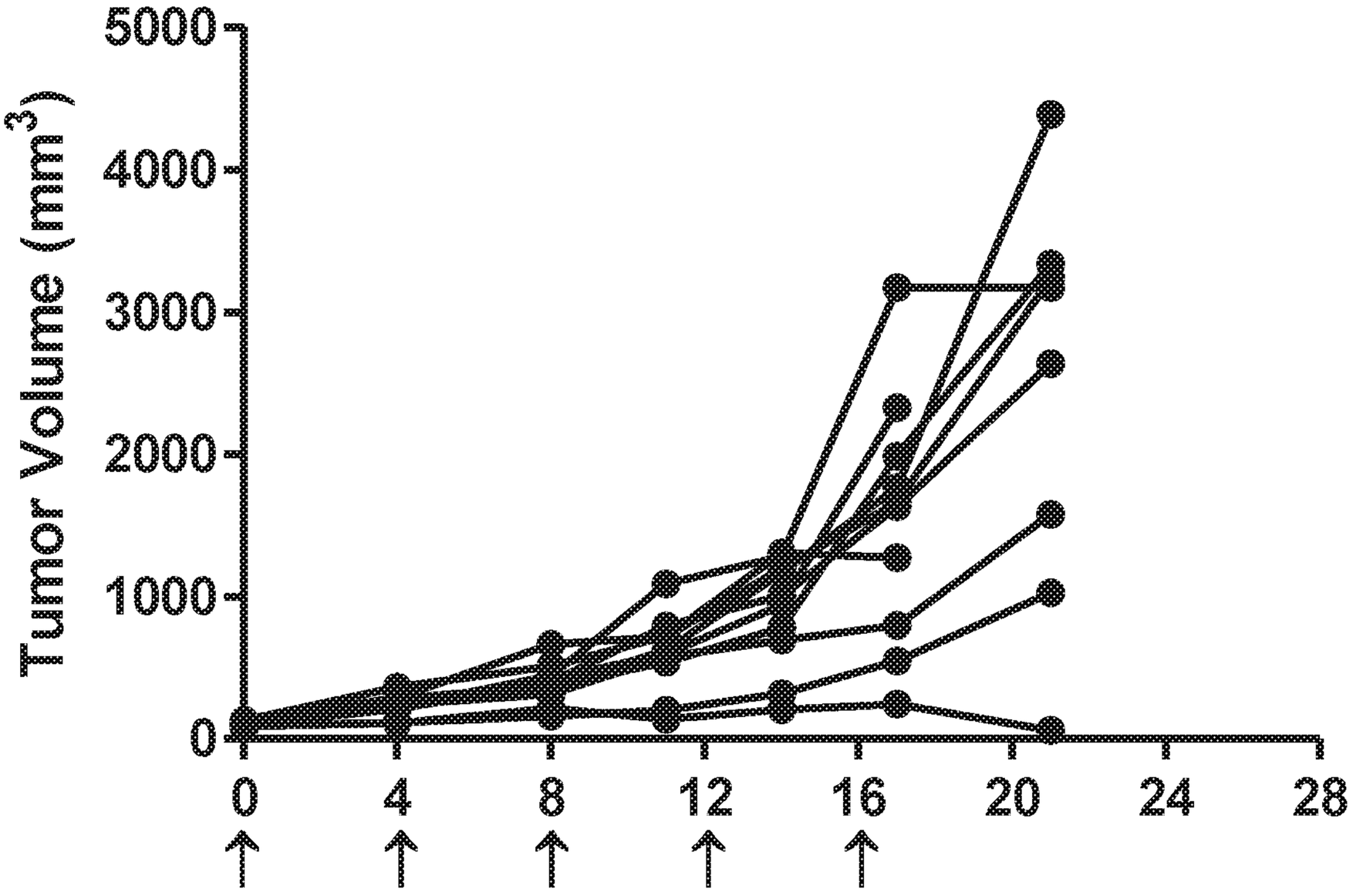

5000
4000
3000
2000
1000
0
Tumor Volume (mm^3)
0
4
8
12
16
20
24
28
Time (days)

FIG. 16

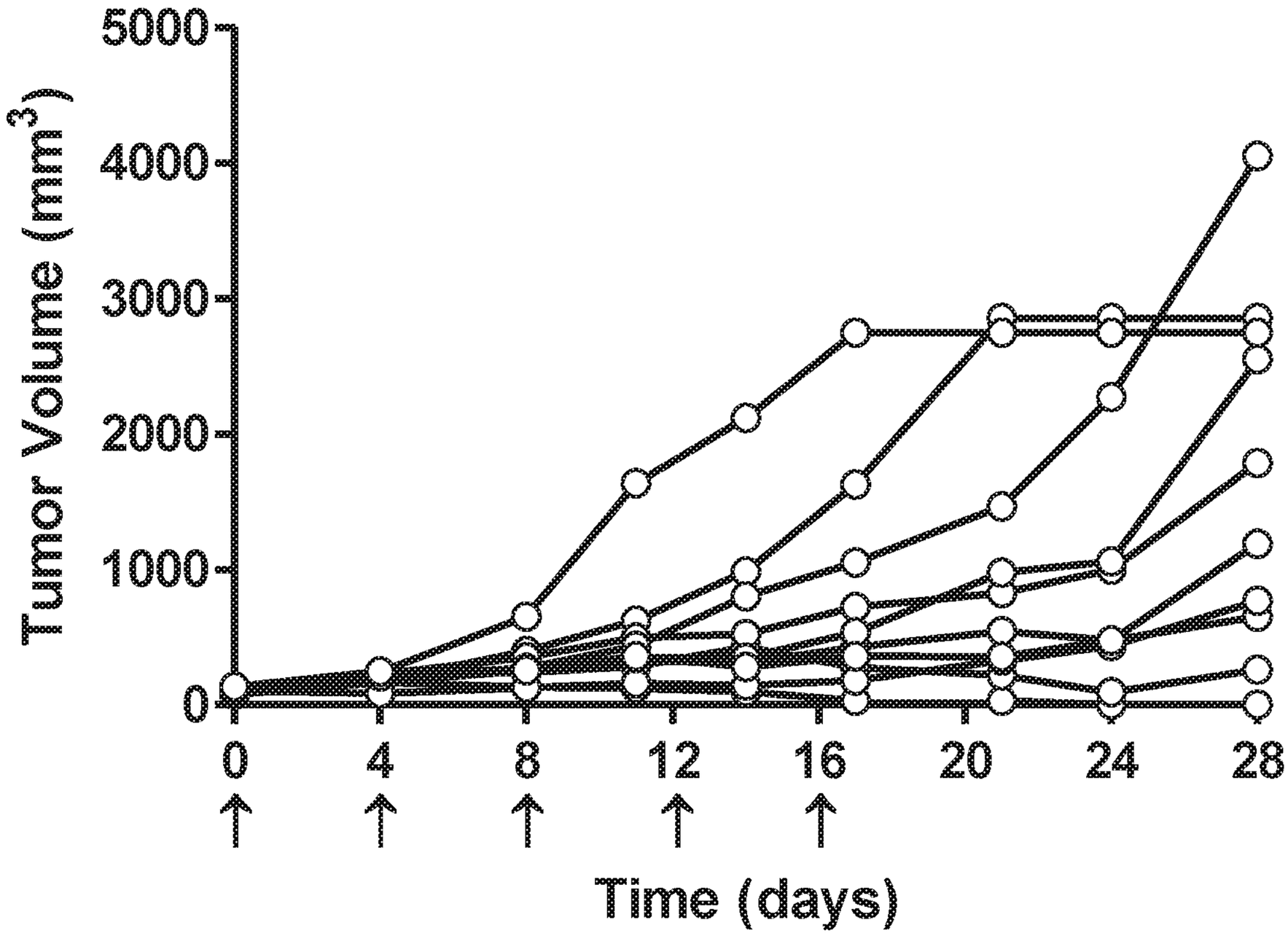
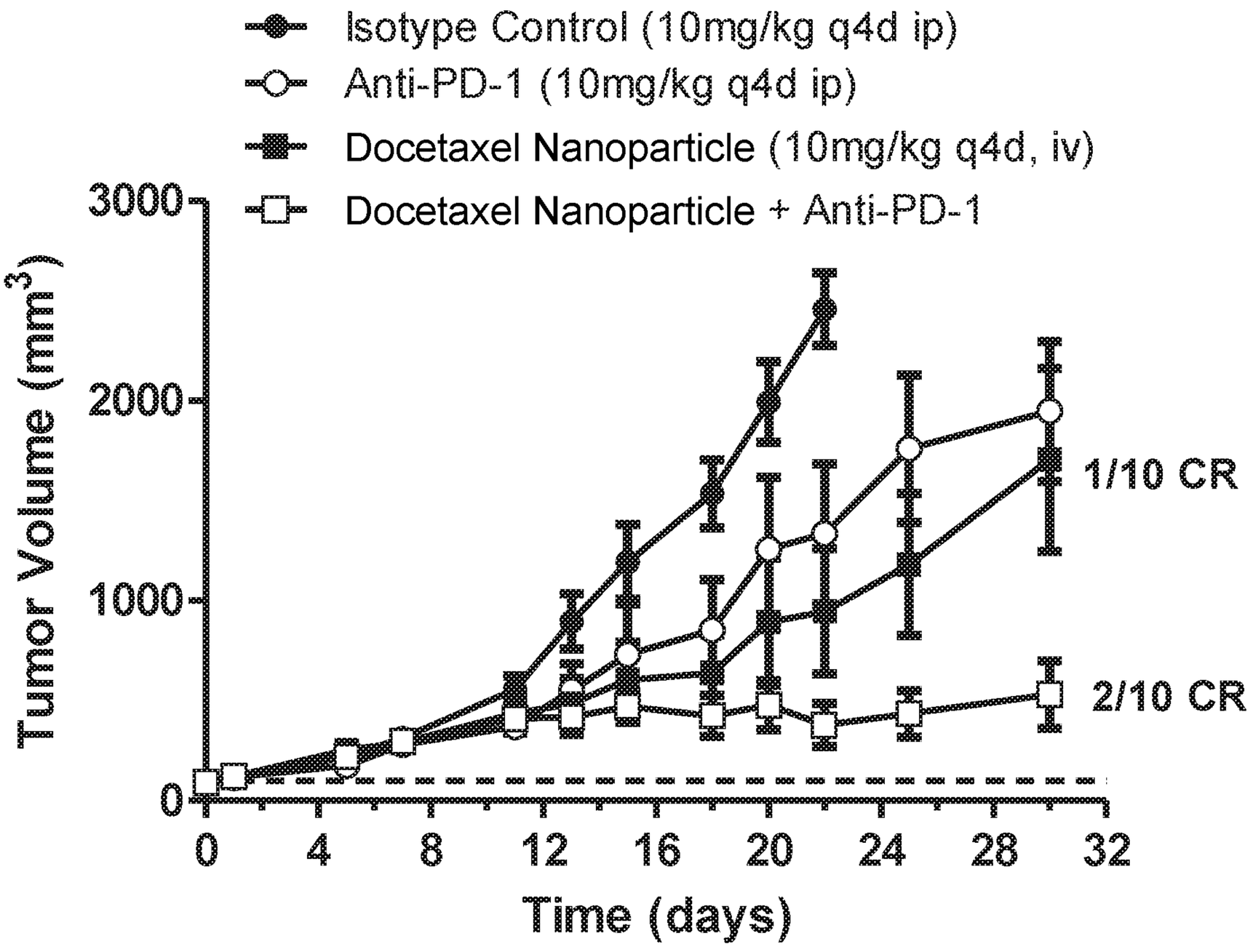

5000
4000
3000
2000
1000
0
Tumor Volume (mm^3)
0
4
8
12
16
20
24
28
Time (days)

FIG. 3


Isotype Control (10mg/kg q4d ip)
Anti-PD-1 (10mg/kg q4d ip)
Docetaxel Nanoparticle (10mg/kg q4d, iv)
Docetaxel Nanoparticle + Anti-PD-1
Tumor Volume (mm3)
3000
2000
1000
0
0
4
8
12
16
20
24
28
32
Time (days)
1/10 CR
2/10 CR