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A Nanoparticle Cocktail: Temporal Release of Predefined Drug Combinations

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Supporting Information

ABSTRACT: A single magic bullet is not enough for treatment of metastatic cancers. However, administration of a combination of free drugs can be extremely challenging because of the inability to control the correct choice of dosages and definitive delivery of the effective drug ratio at the target tissue due to the differences in pharmacokinetics and biodistribution of individual drugs. Here we report an engineered biodegradable polymer containing combination therapeutics that can be self-assembled into a controlled release nanoparticle with abilities to deliver multiple therapeutics in a predefined ratio following temporal release patterns. This platform technology can lead to a rationally designed combination therapy.

Most available anticancer therapies which are directed to an individual molecular target frequently show poor efficacy and resistance due to network robustness, crosstalk, neutralizing actions, and counter-target actions.[1] Advanced cancers include multiple pathways in the disease progression and targeting one of these is an insufficient approach. One such cancer is prostate cancer (PCa) which is the most frequently diagnosed cancer and the second leading cause of cancer death in men in the United States.[2] When PCa progresses in the presence of androgen blockade it is defined as castration-resistant prostate cancer (CRPC).[2−4] Cancer-associated inflammation plays important roles in aggressiveness of PCa.[5] Inhibition of cancer-associated inflammation using anti-inflammatory drugs in combination with chemotherapeutics can be a promising approach for treatment of CRPC.

Unlike single-agent chemotherapy, combination therapy offers modulation of different pathways in cancer cells, maximizing therapeutic efficacy against individual targets and overcoming resistance mechanisms. However, administering a combination of free drugs can be challenging because of the inability to control correct choice of dosages, definitive delivery of appropriate drug ratio at the target tissue, and differences in pharmacokinetic (PK) and biodistribution (bioD) of the individual drugs. Biodegradable controlled release nanoparticles (NPs) have abilities to deliver drugs to the site of interest at an optimum dose.[6−10]

NPs comprising poly(lactide)-*b*-polyethylene glycol (PLA-*b*-PEG) block copolymers are attractive as targeted drug delivery vehicles and confer the advantages of controlled drug release, enhanced stability, and the ability to carry a high payload of drugs. However, this block copolymer PLA-*b*-PEG lacks chemical functionalities for multiple drug/ligand conjugation and does not have the ability to simultaneously deliver both hydrophilic and hydrophobic drugs. The key shortcoming that this polymer lacks functional groups on the aliphatic backbones limits the number of sites for potential conjugation of multiple drugs. Similar limitations apply to most popular biodegradable polymers such as polyglycolide (PGA) and poly(lactide-*co*-glycolide) (PLGA). A handful of backbone functionalized PLA or PLGA derivatives are known in the literature.[11−13] However, backbone-functionalization of polymers is challenging and can potentially lead to immunogenicity, toxicity, and poor biodegradability.

Here we report a unique approach to synthesize PLA with multiple terminal functionalities keeping the backbone close to the FDA-approved skeleton. Numerous functionalities can be introduced in the form of different generation dendrons at the termini of PLA to incorporate an anti-inflammatory drug aspirin (Drug A) and cisplatin prodrug as a chemotherapeutic (Drug B) with a predefined relative stoichiometry to result in the highly functionalized biodegradable polymer $(\text{Drug A})_m$−Dendron 1−PLA−Dendron 2−$(\text{Drug B})_n$, where *m* and *n* are the numbers of Drug A and Drug B, respectively (Figure 1). The blending of

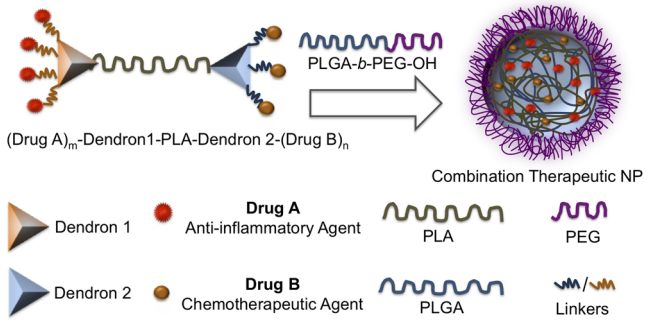

Figure 1. Schematic representation of a combination therapeutic NP from a single polymer containing two drugs.

$(\text{Drug A})_m$−Dendron 1−PLA−Dendron 2−$(\text{Drug B})_n$ with PLGA-*b*-PEG−OH resulted in a NP cocktail containing both anti-inflammatory and chemotherapeutic agents in a predefined ratio (Figure 1). The general applicability of this NP was demonstrated using PCa and cisplatin resistant cancer as models.

An alkyne-functionalized second generation dendron **4** was synthesized from acetonide protected 2,2-bis(hydroxyl-methyl)-propionic acid anhydride (Scheme 1). This dendron allowed the

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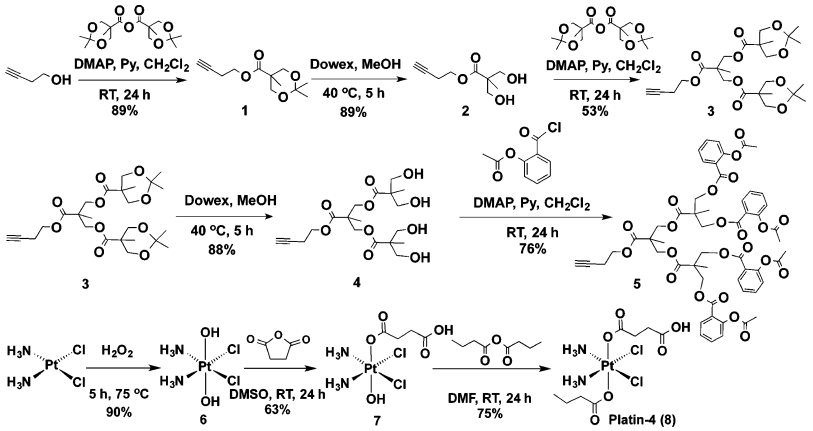
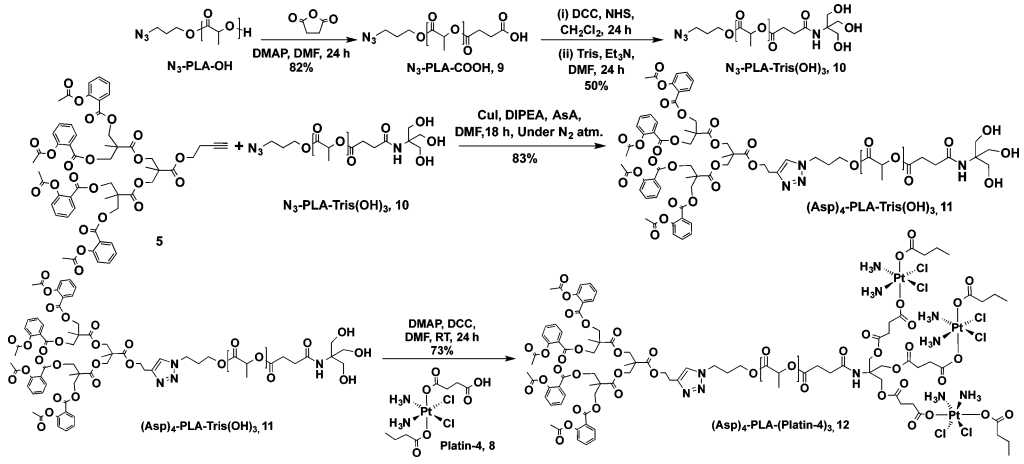
Scheme 1[a]



[a]Synthesis of aspirin conjugated clickable dendron and a Pt(IV) prodrug with −COOH functionality for further reaction with functionalized PLA. DMAP, 4-dimethylaminopyridine; Py, pyridine; RT, room temperature.

Scheme 2[a]



[a]Synthesis of polymers for the construction of both aspirin and Pt(IV) prodrug conjugated functionalized PLA. DCC, *N*,*N*′-dicyclohexylcarbodiimide; NHS, *N*-hydroxysuccinimide; Tris, tris(hydroxymethyl)aminomethane); DIPEA, *N*,*N*-diisopropylethylamine; AsA, ascorbic acid.

loading of four aspirin molecules to result in aspirin-conjugated clickable alkyne-containing dendron **5** (Scheme 1, Supporting Information, Figures S1−S4). Iterative synthesis using **4** can be followed for higher generation dendron to load more numbers of aspirin moieties. For a combination of aspirin with a widely used chemotherapeutic cisplatin,[14] a succinate derivatized Pt(IV) compound Platin-4 (**8**) linked to a butyl chain was constructed (Scheme 1, Figures S5−S7). The −COOH group from the succinic moiety of Platin-4 allowed for its conjugation to another dendron, and the presence of butyl chain prevented any cross reactivity. A carboxy-terminated PLA with a terminal azide N_3−PLA−COOH was developed (**9**, Scheme 2, Figures S8−S9) and further functionalized with tris(hydroxymethyl)aminomethane) or Tris to result in N_3−PLA−Tris$(OH)_3$ with three terminal −OH groups for Platin-4 conjugation (Scheme 2, Figures S9, S10). The resulting N_3−PLA-Tris$(OH)_3$ was "clicked" with alkyne functionalized aspirin containing dendron **5** to result in $(Asp)_4$−PLA−Tris$(OH)_3$ (**11**). This polymer was characterized by ^{1}H, ^{13}C NMR and by gel permeation chromatography (GPC) (Figures S9, S11, S12). Finally $(Asp)_4$−PLA−Tris$(OH)_3$ was coupled with Platin-4 to result in a Janus[15] polymer $(Asp)_4$−PLA−$(Platin\text{-}4)_3$ (**12**) containing both anti-inflammatory and chemotherapeutic agents (Scheme 2, Figures S9, S13−S15). Conjugation of Platin-4 was confirmed from ^{1}H and ^{13}C NMR. Quantification of Pt in the polymer by inductively coupled plasma mass spectrometry (ICP−MS) indicated 71 ± 4 wt % Pt(IV) conjugation in the polymer. Aspirin quantification by high performance liquid chromatography (HPLC) demonstrated 75 ± 5 wt % conjugation efficiency (Figure S15). We previously reported a Pt(IV) prodrug, Platin-*A*,[16] containing both cisplatin and aspirin units; however, in this kind of small molecules, one cannot load multiple copies of both drugs, and incorporation of such water-soluble molecules in a NP matrix is challenging and often encapsulation results in poor loading efficiency.

The blended $(Asp)_4$−PLA−$(Platin\text{-}4)_3$−OH NPs were developed by mixing PLGA-*b*-PEG−OH (Figure S16) with the Janus polymer $(Asp)_4$−PLA−$(Platin\text{-}4)_3$. NP properties and loading of both the drugs were tuned by controlling the ratio of PLGA−*b*-PEG−OH and $(Asp)_4$−PLA−$(Platin\text{-}4)_3$. The NP diameter (*z*-average) increased with higher % feed of $(Asp)_4$−PLA−$(Platin\text{-}4)_3$, and the NP surface was negatively charged due to the presence of −OH groups on the PLGA-*b*-PEG polymer (Figures S17, S18). The effect of percent $(Asp)_4$−PLA−(Platin-

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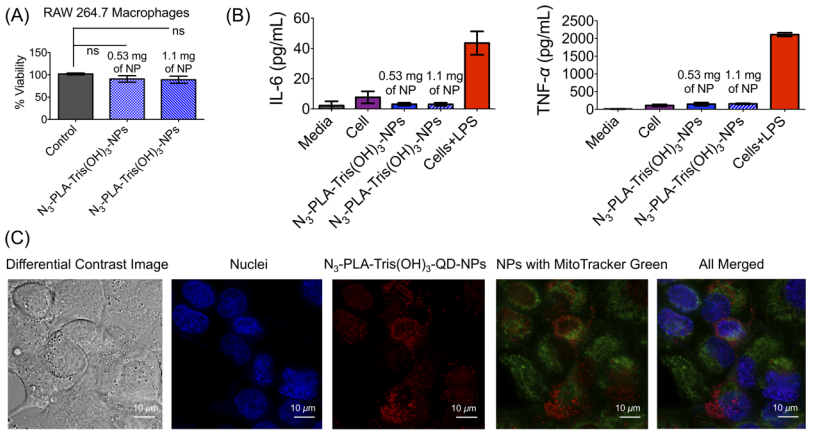

Figure 2. (A) Toxicity of N_3−PLA−Tris(OH)$_3$−NPs in RAW 264.7 macrophages at two different concentrations as determined by the MTT assay. NP treatment was carried out for 24 h. All statistical analyses were performed using GraphPad Prism software performing a one-way analysis of variance (ANOVA) and nonparametric analyses followed by the Tukey post-test: ns, nonsignificant. (B) Effects of different concentrations of N_3−PLA−Tris(OH)$_3$−NPs in the absence or presence of LPS in RAW 264.7 macrophages by assessing IL-6 and TNF-α: LPS, lipopolysaccharide (C) Uptake of N_3−PLA−Tris(OH)$_3$−QD NPs in PCa DU145 cells by live cell imaging after 2 h incubation period.

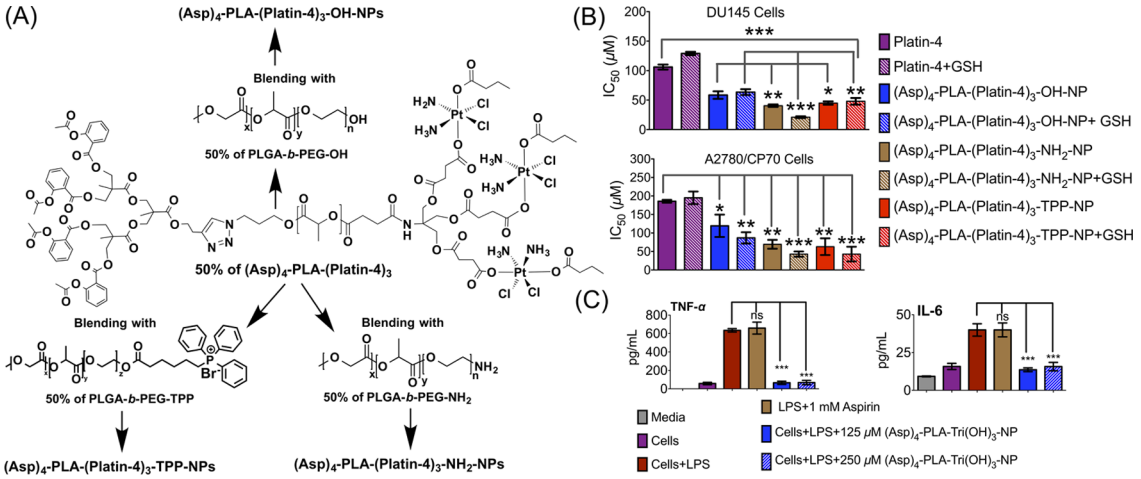

Figure 3. (A) Construction of (Asp)$_4$−PLA−(Platin-4)$_3$−OH−NPs, (Asp)$_4$−PLA−(Platin-4)$_3$−NH$_2$−NPs, (Asp)$_4$−PLA−(Platin-4)$_3$−TPP−NPs from different polymers. (B) Cytotoxicity of (Asp)$_4$−PLA−(Platin-4)$_3$−OH−NPs, (Asp)$_4$−PLA−(Platin-4)$_3$−NH$_2$−NPs, (Asp)$_4$−PLA−(Platin-4)$_3$−TPP-NPs and comparison of activity with Platin-4 in prostate cancer DU145 and cisplatin resistant ovarian cancer A2780/CP70 cells. (***) $P < 0.001$; (**) $P = 0.001$−0.01; (*) $P = 0.01$−0.05. (C) Abilities of (Asp)$_4$−PLA−Tris(OH)$_3$−NPs to reduce inflammation in LPS-stimulated RAW 264.7 macrophages. (***) $P < 0.001$; ns, nonsignificant. All statistical analyses were carried out using GraphPad Prism software performing a one-way ANOVA and nonparametric analyses followed by the Tukey post-test.

4)$_3$ feed on % loading and % encapsulation efficiency (%EE) indicated that both Pt(IV) and aspirin loading increased up to 50% feed of (Asp)$_4$−PLA−(Platin-4)$_3$ and then declined (Figures S17, S19). Transmission electron microscopy (TEM)-based morphology studies indicated that these NPs are spherical, homogeneous at a lower % feed of (Asp)$_4$−PLA−(Platin-4)$_3$. As the % feed of the Janus polymer increased, the NP diameter increased and NPs became heterogeneous (Figure S17). For anti-inflammatory studies, NPs were prepared by blending (Asp)$_4$−PLA−Tris(OH)$_3$ with PLGA-*b*-PEG−OH or N_3−PLA−Tris(OH)$_3$ with PLGA-*b*-PEG−OH (Figures S20, S21). Characterization of NPs prepared on different days from (a) a given batch of polymers, and (b) different batches of polymers indicated high reproducibility. Stability studies for 7 days in water or 10% fetal bovine serum-water at 4 and 37 °C indicated high stability of the blended 50%-(Asp)$_4$−PLA−(Platin-4)$_3$−OH−NPs (Figures S22, S23). On the basis of dynamic light scattering, TEM, % loading, % EE, and stability details, we decided to use 50%-(Asp)$_4$−PLA−(Platin-4)$_3$−OH−NPs in our further studies.

Release of Pt(IV) and aspirin from (Asp)$_4$−PLA−(Platin-4)$_3$−OH−NPs was investigated by dialyzing the NPs against phosphate buffered saline of pH 7.4 at 37 °C and analyzing the released Pt and aspirin by ICP-MS and HPLC, respectively. Well-controlled and temporal release kinetics of Pt and aspirin were observed with Pt being released at a much faster rate compared to aspirin (Figures S24, S25). This temporal release profile arises due to the presence of aromatic ester linkages between aspirin and the polymer, whereas Platin-4 is conjugated to the polymer chain via aliphatic ester linkages, in addition Pt can also get released via reduction. Stimuli responsive release in the presence of sodium ascorbate to accelerate release of Pt(IV) by reduction and esterase to cleave the ester bonds to aspirin indicated that both the drugs can be released at a much faster rate under the influence of stimuli (Figures S24, S25). The presence of the reducing agent accelerated Pt release, and the esterase only released aspirin at a faster rate indicating temporal stimuli

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responsiveness of these NPs. The ability of Platin-4 to form an adduct with guanine bases was studied by carrying out the reaction with 2′-deoxyguanosine 5′-monophosphate (5′-dGMP) in the presence of sodium ascorbate, demonstrating formation of $[Pt(NH_3)_2(5'\text{-dGMP-N7})_2]$ adduct (m/z = 922, Figure S26).

Toxicity of the precursor polymer was determined by using 50%-N_3−PLA−Tris(OH)$_3$−NPs in RAW 264.7 macrophages at different concentrations (Figure 2A). These studies indicated that this polymer is nontoxic. Assessment of 50%-N_3−PLA−Tris(OH)$_3$−NPs in RAW 264.7 cells indicated no significant secretion of tumor necrosis factor alfa (TNF-α) or interleukin (IL)-6 supporting that this NP platform is nonimmunogenic (Figure 2B). To provide insight on the cellular uptake of the blended NPs, we coencapsulated a hydrophobic polymer conjugated to fluorescent quantum dot (QD), PLGA-*b*-PEG−QD[8] (Figure 2C). NP uptake studies in PCa DU145 cells indicated that the NPs are taken up in the cytosolic matrix in ∼2 h. That these NPs delivered the drugs in their conjugated forms is supported by the fact that only small fractions of Platin-4 or aspirin are released from the NPs in 2 h and substantial amounts of NPs are taken up by the cells in 2 h.

Cytotoxic activity of (Asp)$_4$−PLA−(Platin-4)$_3$−OH−NPs (Figure 3A) in DU145 cell lines indicated that when Platin-4 was delivered using the NPs, there was a decrease in its inhibitory concentration-50 (IC_{50}) in DU145 cells (Figure 3B, Figure S27). Similarly, in cisplatin resistant ovarian cancer A2780/CP70 cells (Asp)$_4$−PLA−(Platin-4)$_3$−OH−NPs demonstrated higher efficacy compared to Platin-4 and the cytotoxicity of the NPs was enhanced in the presence of glutathione (GSH) (Figure 3B). A comparison of IC_{50} values of (Asp)$_4$−PLA−(Platin-4)$_3$−OH−NPs with those of cisplatin in both A2780/CP70 and DU145 cells indicated that the NPs are less active compared to cisplatin (Figure S28). To understand whether less efficacious activity of (Asp)$_4$−PLA−(Platin-4)$_3$−OH−NPs is related to low uptake of the NPs, we constructed (Asp)$_4$−PLA−(Platin-4)$_3$−NH_2−NPs and (Asp)$_4$−PLA−(Platin-4)$_3$−TPP−NPs by blending (Asp)$_4$−PLA−(Platin-4)$_3$ with PLGA-*b*-PEG−NH_2 and PLGA-*b*-PEG−triphenylphosphonium (TPP) cation polymer,[8] respectively, with the rationale that cationic NPs demonstrate enhanced cellular uptake (Figures 3A and S29, S30). Cytotoxicity evaluation of these three different NPs indicated that less efficacious uptake of (Asp)$_4$−PLA−(Platin-4)$_3$−OH−NPs partially contributed to low cytotoxic behavior as the efficacy was enhanced with the cationic NPs in both the cell lines (Figure 3B). Cytotoxicity of the cationic NPs were further enhanced by the presence of GSH supporting that slow release of Pt(IV) is also responsible for the less efficacy (Figure 3B) of this NP platform. Slow release of the drugs from the polymer chains might play important roles in demonstrating controlled delivery of the drugs and hence higher therapeutic potency when administered *in vivo*.

Pro-inflammatory cytokines TNF-α and IL-6 are traced in the vicinity of tumor tissue. We therefore investigated the effects of (Asp)$_4$−PLA−Tris(OH)$_3$−NPs on production of these two cytokines when RAW 264.7 macrophages were stimulated with LPS. The NPs showed reduction in IL-6 and TNF-α in LPS stimulated macrophages. Aspirin at a high concentration did not show such efficient anti-inflammatory properties compared to the blended NPs (Figure 3C). Control N_3−PLA−Tris(OH)$_3$-NPs did not inhibit LPS-induced IL-6 and TNF-α production supporting that the anti-inflammatory activities of (Asp)$_4$−PLA−Tris(OH)$_3$-NPs arise from aspirin and rules out the possibility of simple binding of LPS with the NPs (Figure S31).

Currently available controlled release NPs of PLA, PGA, and their copolymer PLGA lack the ability for controlled delivery of a combination of two drugs at a correct therapeutic ratio. Our technology using a functionalized biodegradable polymer, which can load both hydrophobic and hydrophilic drugs at a predefined stoichiometric ratio, and all chemical conjugation steps before NP formulation, provides an advanced platform. The utility of the current multidrug NP approach could make a major impact for diseases requiring combination therapies.

■ ASSOCIATED CONTENT

Supporting Information

Details of materials and instrumentation, synthesis and characterization of monomers, polymers, additional experimental details and data. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b03078.

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Notes

The authors declare no competing financial interest.

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