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Camouflaging Nanoparticles for Ratiometric Delivery of Therapeutic Combinations

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Abstract

Combination therapy is a common clinical practice in management of malignancies. Synergistic therapeutic outcomes are achieved only when tumor cells are exposed to drugs in an optimal ratio and sequence; therefore, carriers coencapsulating multiple drugs are widely pursued for their coordinated delivery. However, it is challenging to co-load drugs with different physicochemical properties in a single carrier with specific ratios. It is not even beneficial to load them in one carrier if they need be released at different times. Herein, we propose to load drugs into chemically compatible carriers separately, equalize different carriers by a simple, rapid and versatile camouflage technique based on natural polyphenol tannic acid (TA) and administer them in desirable ratios and sequences. To demonstrate this potential, different nanoparticles (NPs) with different charges and material basis, such as polymeric (carboxyl-terminated or amine-terminated cationic polystyrene NPs or poly(lactic-co-glycolic acid (PLGA) NPs), inorganic (mesoporous silica NPs, MSNs), and liposomal NPs, are camouflaged with TA-layers and further modified with folate-conjugated polyethylene glycol to aid in the delivery to tumors. The camouflaged NPs show similar physicochemical properties and interactions with KB cells, despite the difference in core platforms, and their mixtures interact with common cell targets in a ratiometric manner. In KB-tumor bearing mice, the camouflaged PLGA NPs and MSNs show near-perfect colocalization in tumors. These results support that TA helps equalize different NPs with high versatility and enables their ratiometric delivery to common targets. This approach can relieve technical challenges in ratiometric co-delivery or sequential delivery of therapeutic agents with distinct physicochemical properties.

Keywords

Combination therapy; coordinated delivery; surface camouflage; nanoparticles; tannic acid assembly

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In chemotherapy of cancer, it is common to use combinations of drugs.^{1–3} The rationale of combination therapy is multifaceted. First, cancer cells carry multiple abnormalities, which may not be addressed by a single drug. Thus, cytotoxic drugs with different molecular targets are often combined to avoid the development of drug resistance.² For example, paclitaxel and gemcitabine, which are inhibitors of microtubule depolymerization and DNA synthesis, respectively, are used together as the first-line treatment of metastatic pancreatic cancer.⁴ In addition, cancer cells treated with a single drug can use alternative survival pathways and become resistant to the treatment.⁵ Therefore, experimental studies have used combinations of chemotherapy and gene therapeutics targeting various aspects of tumor progression.⁶ For example, siRNA targeting P-glycoprotein has been used in combination with paclitaxel and doxorubicin to overcome drug resistance of tumor cells.^{7, 8} Emerging new therapeutics can also benefit from combination therapy. Co-administration of a transforming growth factor- β inhibitor facilitates cytotoxic T-cell infiltration into tumors and significantly enhances immune checkpoint blockade therapy of mammary carcinoma.⁹ A recent study also finds that the microenvironment limits therapeutic effects of gemcitabine by harboring bacteria that produce drug-inactivating enzymes.¹⁰ In this case, co-administration of antibiotics is warranted to enhance gemcitabine-based chemotherapy.

Optimal ratio or dosing sequence of combined drugs are critical to synergistic effects.^{11–13} With conventional drug cocktails, it is however difficult to deliver the drug combination in a spatiotemporally optimal manner due to differential pharmacokinetics and biodistribution patterns of the component drugs. Therefore, synergistic combinations identified *in vitro* do not always translate to clinical benefits.¹⁴ For coordinated delivery of drug combinations, it may thus be favorable to formulate the drugs in a single nanocarrier to unify their pharmacokinetics and deploy them at target sites.¹¹ However, drugs with drastically different physicochemical properties are rather difficult to load in a single nanocarrier with high efficiency, let alone in a specific ratio.¹⁵ Moreover, if the drugs are to act on different cells in target tissues¹⁶ and/or show a synergistic effect only when provided in a specific order,^{12, 13} it is not even desirable to load them in a common carrier. Alternatively, drugs may be separately loaded in chemically compatible carriers that allow for optimal drug loading and administered in desirable ratios and sequences. This will not only reduce technical burdens involved in formulation development but also help clinicians to adjust the dose of each agent and dosing schedule according to the patient's responses to ongoing treatments. The challenge is to ensure that the carriers arrive at the same target tissues. Different carriers that are unable to co-localize in the target site may attenuate the synergistic effects of drug combinations or even cause antagonistic effects due to suboptimal drug ratios.¹⁷ To achieve colocalization of different nanocarriers, it is critical to control their surface properties, which dictate the interactions with serum proteins and cell populations, thereby the pharmacokinetics and biodistribution of the carriers.^{18–21}

We hypothesize that camouflaging different nanocarriers with a common surface may help equalize their biological behaviors, facilitating coordinated delivery of drug combinations with different physicochemical properties. However, it is technically challenging to modify the surfaces of different drug carriers with the same material due to their difference in chemical reactivity. In addition, chemical reactions used for surface modification of nanocarriers typically involve complex procedures and exhaustive purification steps,^{22–25}

which can be detrimental to the integrity of the nanocarriers and the production efficiency. Therefore, we use a simple, rapid, and versatile surface modification method involving tannic acid (TA), a natural polyphenol, for equalizing nanocarrier surface. TA can self-assemble to form a thin film on solid platforms irrespective of their composition and surface reactivity,^{26, 27} with an optional aid of Fe^{3+} ,^{28, 29} effectively masking the underlying substrate. The TA and Fe-TA coordination complexes (pTA) can accommodate thiol- or amine-terminated functional ligands on the NP surface through Michael addition or Schiff base reactions.^{26, 30, 31} (p)TA can also interact with the ligands via additional mechanisms such as electrostatic interactions, hydrogen bonding and hydrophobic interactions.³² The (p)TA coating can be performed on practically any platforms with high efficiency and speed in neutral aqueous solutions; therefore, it is ideal for modifying various nanocarriers, including those unable to survive prolonged exposure to reactive conditions or exhaustive purification processes. Moreover, TA is biodegradable in physiological conditions due to the abundant ester groups³³ and has been well tolerated in parenteral applications.^{34, 35}

In this study, we take advantage of the versatility, efficiency, and safety of TA to equalize the surfaces of various NPs and enable a coordinated delivery of different drug combinations. To test whether TA can help camouflage a wide range of NPs, we modify NPs with different charges and material basis (including common drug carriers), such as polymeric (PLGA and polystyrene (PS)), inorganic (mesoporous silica), and liposomal NPs by (p)TA, followed by additional modification with folate-conjugated polyethylene glycol (pFol), a model ligand with well-defined interactions with folate receptor-positive cancer cells (Table 1). The physicochemical properties of the camouflaged NPs and their interactions with folate receptor-overexpressing KB cells are examined to confirm the surface modification. Selected mixtures of camouflaged NPs are then evaluated *in vitro* and *in vivo* to demonstrate co-delivery to common targets.

The efficiency and versatility of TA-mediated surface modification was first tested with PS NPs (carboxyl-terminated anionic PS NPs, PS_{COOH} or amine-terminated cationic PS NPs, PS_{NH_2}) as a model platform. In the absence of Fe^{3+} , PS_{COOH} acquired TA corresponding to 1.06 ± 0.04 wt% after 2 h incubation with TA (4 mg/mL per 1 mg/mL PS_{COOH} ; i.e., TA/ PS_{COOH} feeding ratio of 400 wt%). Additional incubation up to 24 h did not further increase the TA content (Figure S2). The particle size of PS_{COOH} -TA (PS_{COOH} coated with TA) was not significantly different from bare PS_{COOH} (Figure S2 and Table S1). On the other hand, pTA (TA-Fe assembly) coated the PS_{COOH} surface much more efficiently than TA alone (in <1 min), achieving stoichiometric increase of pTA content with the TA feed (Figure S3). The order of TA and Fe^{3+} addition apparently mattered. When TA was added before Fe^{3+} , 97% of the fed TA deposited on PS_{COOH} , whereas only 63% of the fed TA deposited on the PS_{COOH} surface with the opposite order. It is possible that TA added first creates a pre-adsorbed TA layer on PS_{COOH} surface that complexes with subsequently added Fe^{3+} and crosslinks with additional TA molecules via the Fe^{3+} . The opposite order is likely to create a high initial Fe^{3+} -to-TA ratio, leading to rapid formation of pTA complexes in the bulk solution, which may or may not adsorb to the NP surface.^{28, 29} The suspension of PS_{COOH} -pTA showed dark blue color indicating the presence of pTA complex (Figure S4, S5).^{28, 36} TEM (Figure S4) showed no visible pTA self-assemblies (Figure S6), indicating that they deposited on the NP surfaces. The particle size measured by DLS apparently increased with

the TA added to form $\text{PS}_{\text{COOH}}\text{-pTA}$ (Table S2). However, TEM images did not show corresponding size difference (Figure S4a), indicating that the size increase may reflect mild aggregation in the buffer in which the particle size was measured. The zeta potential of PS_{COOH} remained strongly negative irrespective of the pTA coating (Table S2) because both bare PS_{COOH} and pTA (due to galloyl groups³⁷) have negative charges. Fe^{3+} does not significantly reduce the negative charge because it is mostly located in between TAs. Moreover, Fe^{3+} can increase the ionization of TA, rather enhancing its negative charge.²⁹

PS_{NH_2} were more efficiently coated with TA than PS_{COOH} . Fe^{3+} was not required for the coating of PS_{NH_2} , as anionic TA was readily attracted to cationic PS_{NH_2} via electrostatic interactions. Fe^{3+} may rather reduce the ionization of amine termini of PS_{NH_2} ²⁹ and interfere with electrostatic adsorption of TA; therefore, Fe^{3+} was omitted in the preparation of TA-coated PS_{NH_2} ($\text{PS}_{\text{NH}_2}\text{-TA}$) (Figure S4). The TA content in the optimized $\text{PS}_{\text{NH}_2}\text{-TA}$ was 12.6 ± 0.06 wt% (Figure S7). The TA coating completed in 0.5 h, and no further changes in zeta potential and TA content occurred with additional incubation (Figure S7). $\text{PS}_{\text{NH}_2}\text{-TA}$ in bicine buffer (100 mM, pH 7.8) showed a strong negative charge (-35.6 ± 1.0 mV) and stayed well suspended in 10% FBS for 24 h without changing the size (Figure S4).

Lip, M, and P NPs were modified with pTA just as efficiently. Similar to PS_{COOH} , these NPs showed dark blue color after coating with pTA (Figure 1, Figure S8) and size increase after the pTA modification (Table 1, Table S3). All TA-coated NPs showed similarly negative charges (-26.7 to -30.3 mV) despite the large difference of bare NPs (Lip: -5.0 mV; P: -10.6 mV; and M: -33.7 mV), indicating that they acquired similar surface layers. The TA content in Lip-pTA and P-pTA increased in 1:1 ratio to the TA feed per NPs (Figure S9), which means complete incorporation of the used TA. However, the TA content in M-pTA was half the Lip-pTA and P-pTA's at the same TA feed per NPs, likely due to the shortage of continuous surface. The MSNs soaked in PEG solution (M_{PEG}) incorporated TA more efficiently than bare M, forming $\text{M}_{\text{PEG}}\text{-pTA}$ with a higher TA content (7.7% vs 3.5% of M-pTA) at a TA feed ratio of 8%. As shown in TEM images (Figure 1 vs. Figure S10), $\text{M}_{\text{PEG}}\text{-pTA}$ and $\text{M}_{\text{PEG}}\text{-pTA-pFol}$ appeared darker than M-pTA, which suggests that PEG filled the pores of MSNs to form M_{PEG} with continuous surface favorable for TA coating. Indeed, M_{PEG} , unlike bare M, showed a similar coating efficiency as Lip and P. Therefore, M_{PEG} was used for further functionalization.

The (p)TA-coated NPs ($\text{PS}_{\text{COOH}}\text{-pTA}$, $\text{PS}_{\text{NH}_2}\text{-TA}$, Lip-pTA, P-pTA and $\text{M}_{\text{PEG}}\text{-pTA}$, collectively called NP-(p)TA) were functionalized with folate-PEG conjugates (pFol, 5000 Da) to ensure comparable interactions with cancer cells upon extravasation and arrival at tumors. pFol was chosen as a model ligand due to their well-defined interactions with folate receptor-positive cancer cells. All NPs (NP-(p)TA-pFol) were readily modified with pFol irrespective of the core platforms (Table 1). All showed less negative charge compared to NP-(p)TA after simple incubation with pFol, indicating the shielding effect of PEG groups in pFol. The particle size of NP-pTA-pFol was comparable to or even smaller than NP-pTA due to pFol layer helping to disperse the NPs. As shown with P-pTA-pFol and $\text{M}_{\text{PEG}}\text{-pTA-pFol}$, the pFol content in each NP increased with pFol feed, reaching saturation at higher feeds (Figure 1b). Despite the evidence of pFol conjugation, there was no significant difference between NP-pTA-pFol and NP-pTA in TEM images (Figure 1a), similar to the NPs

functionalized via polydopamine.^{38–40} The conjugated pFol remained stable on the NP surface in 50% FBS, a medium used to mimic blood. As shown in Figure S11, pFol was detectable with P-pTA-pFol and M_{PEG}-pTA-pFol incubated in 50% FBS at 37 °C for 24 h. This suggests that intermediate pTA layer may be stable in circulation and able to maintain pFol on NP surface at least for 24 h.

To confirm the functionality of the surface-bound pFol, we incubated KB cells with the fluorescently-labeled *NP*-(p)TA, *NP*-(p)TA-pFol, and their bare NP counterparts for 3 h and determined their fluorescence intensity as a measure of cell-NP interactions (Figure 2a). KB cells incubated with PS_{COOH}-pTA showed slightly higher fluorescence intensity than those with PS_{COOH}, likely due to the adhesive pTA layer.⁴¹ The fluorescence increase was not reversed by extra folate, indicating that the interaction of PS_{COOH}-pTA with KB cells was non-specific. Incubation with PS_{COOH}-pTA-pFol led to significant increase in KB cell fluorescence, which was inhibited by extra folate. This result verifies that PS_{COOH}-pTA-pFol interacted with KB cells via folate receptors. It also indicates that the interaction of PS_{COOH}-pTA-pFol with KB cells is mainly governed by the outmost layer (pFol) and the effect of intermediate pTA layer is minimal. The cells incubated with PS_{NH2}-TA showed much lower fluorescence intensity than those with PS_{NH2}. PS_{NH2} may have interacted with cells via their positive charges, whereas PS_{NH2}-TA with the neutralized surface showed relatively low interactions with KB cells. Extra folate did not affect the fluorescence intensity, indicating that the interactions of PS_{NH2} or PS_{NH2}-TA with KB cells, regardless of the extent, were irrelevant to folate receptors. Similar to PS_{COOH}-pTA-pFol, the incubation with PS_{NH2}-TA-pFol resulted in significant increase in cellular fluorescence compared to PS_{NH2}-TA, which was reduced by extra folate to the level comparable to those treated with PS_{NH2}-TA. P and M_{PEG} showed similar patterns as PS_{COOH} (Figure 2b). P-pTA showed greater cellular interactions than P, unaffected by extra folate. Both P-pTA-pFol and M_{PEG}-pTA-pFol showed greater interactions with KB cells than P-pTA and M_{PEG}-pTA, respectively, and extra folate brought the cellular fluorescence to the same level as those with P-pTA and M_{PEG}-pTA. Confocal microscopy also demonstrated that P-pTA-pFol and M_{PEG}-pTA-pFol interacted with KB cells substantially better than their bare NP counterparts at the same concentration and the majority of them were present in the cytoplasm (Figure 2c). These results support that PS_{COOH}, PS_{NH2}, P, and M were efficiently modified with pFol via (p)TA and underwent folate receptor-mediated interactions with KB cells.

We then examined whether the flexible surface modification would help co-localize different NPs in KB cells. A mixture of PS_{COOH} and PS_{NH2}, polystyrene NPs with opposite surface charges, was first tested with and without pFol modification. Both PS_{COOH}-pTA-pFol and PS_{NH2}-TA-pFol interacted with KB cells via folate receptors; therefore, it was expected that the two NPs, if incubated together, would compete for KB cells. PS_{NH2}-TA-pFol and PS_{COOH}-pTA-pFol were prepared in comparable sizes and zeta potentials (Table 1), and their mixture (or a mixture of their bare counterparts) were incubated with KB cells to examine how one type of NPs affects the other's interaction with the cells. Specifically, KB cells were incubated with a fixed amount of fluorescently labeled PS_{NH2}-TA-pFol (or PS_{NH2}) and an increasing amount of non-fluorescent ⁰PS_{COOH}-pTA-pFol (or ⁰PS_{COOH}) to show whether the cellular fluorescence (KB cells interacting with PS_{NH2}-TA-pFol or PS_{NH2}) decreased due to the competing ⁰NPs. When incubated alone with KB cells, PS_{NH2} and

PS_{NH2}-T-pFol induced linear increase in the cell fluorescence by 120 µg/mL and 350 µg/mL, respectively, whereupon the fluorescence increase showed a brief lag and then resumed (Figure S12). The lag is likely to indicate initial saturation of binding sites, and the subsequent fluorescence increase may result from the uptake of initially bound NPs and the release of the available binding sites. With the PS_{NH2} and PS_{NH2}-T-pFol fixed at each saturation concentration, potential competitors were added to increase their percentage in the total NP population (Figure 3a). PS_{NH2} binding to KB cells was not affected by °PS_{COOH} (Figure S13), indicating that the two NPs did not compete with each other due to the large difference in surface properties. Likewise, additional °PS_{COOH} did not alter the extent PS_{NH2}-TA-pFol interacted with KB cells, verifying the lack of competition. PS_{NH2}-TA-pFol interaction with KB cells linearly decreased with the amount of °PS_{NH2}-TA-pFol, which served as a positive control (known competitor). Reflecting the similarity to PS_{NH2}-TA-pFol in size and surface properties, °PS_{COOH}-pTA-pFol induced the decrease of PS_{NH2}-TA-pFol interaction with KB cells in a concentration-dependent manner. At a relatively low concentration (<20% of the total NP population), °PS_{COOH}-pTA-pFol did not compete effectively with PS_{NH2}-TA-pFol, suggesting a slight difference from PS_{NH2}-TA-pFol, such as the underlying TA layer (pTA vs. TA) and pFol content. Nevertheless, when added to >20% of the total NP population, °PS_{COOH}-pTA-pFol induced a linear decrease of PS_{NH2}-TA-pFol interaction with KB cells, similar to °PS_{NH2}-TA-pFol, indicating that °PS_{COOH}-pTA-pFol and PS_{NH2}-TA-pFol had functionally similar surfaces and interacted with KB cells in the same mechanism. These results demonstrate that NPs with vastly different surfaces, such as PS_{COOH} and PS_{NH2}, can be camouflaged to functionally comparable NPs by TA-mediated surface modification to target common cell populations.

Based on this premise, we then tested whether P and M, as potential carriers of hydrophobic and hydrophilic drugs, respectively, can be delivered to KB cells in a ratiometric manner through the TA-mediated surface modification. P-pTA-pFol and M_{PEG}-pTA-pFol with comparable size and surface charge (Table 1) were mixed at specific ratios (100 µg/mL of M_{PEG}-pTA-pFol and varying amounts of P-pTA-pFol) and incubated with KB cells for 2 h to determine the amount of each NPs internalized by the cells (Figure 3b–e, Figure S14). Without surface modification, both P and M showed minimal uptake by the cells (Figure 3b). In contrast, P-pTA-pFol and M_{PEG}-pTA-pFol were taken up by KB cells in the amounts precisely reflecting the NP feeds (Figure 3c), revealing a 1:1 relationship between the NP feed ratio and the intracellular NP ratio (Figure 3d). Consistently, confocal microscopy showed little signals of unmodified P and M in KB cells (Figure 3e). However, P-pTA-pFol and M_{PEG}-pTA-pFol showed much brighter intracellular signals and efficiently co-localized in the cells (Figure 3e, Figure S15) with a Pearson's correlation coefficient (R) of 0.76 (perfect colocalization: R = 1; no colocalization: R = 0; and perfect exclusion: R = -1). No crosstalk was observed between the green signal of M_{PEG}-pTA-pFol and the red signal of P-pTA-pFol (Figure 2c), verifying that the overlap of two colors indicates NP co-localization. These results support that P-pTA-pFol and M_{PEG}-pTA-pFol can be delivered to target cells in a desired ratio.

For quantitative analysis of the NP distribution *in vivo*, fluorescently-labeled P-pTA-pFol and M_{PEG}-pTA-pFol (or P and M, their unmodified counterparts) were administered individually or in combination to KB tumor-bearing mice by tail-vein injection. Their

distribution in tumor and liver was quantified after 8 or 24 h (Figure 4, Figure S16). Individually administered, P-pTA-pFol and M_{PEG}-pTA-pFol showed similar levels of distribution in both tumor and liver, as expected from the similarity in sizes and surface properties of the two NPs. Unmodified P and M also did not show significant difference from each other in tumor or liver accumulation; however, the amount delivered to tumors was much less than those of pFol-modified counterparts. The low tumor accumulation may be attributed to the short circulation half-lives of P (< 1 min⁴²) and M (~15 min⁴³). The relatively high tumor accumulation of P-pTA-pFol and M_{PEG}-pTA-pFol suggests that pFol modification successfully extended their circulation half-lives and facilitated the interactions with KB tumors *in vivo*. A consistent trend was observed when the pairs were administered in combination. At 8 h post-injection, P-pTA-pFol and M_{PEG}-pTA-pFol administered in 1:1 weight ratio were detected in tumor and liver in comparable quantities. The amounts of the pFol-modified NPs in tumors were significantly greater than those of unmodified NPs, while the levels in the liver were similar irrespective of pFol modification. This trend persisted till 24 h post-injection. These results show that pFol modification enhances tumor delivery of P and M to similar extents, offering the potential to codeliver drug combinations in two different carriers.

To examine whether the co-administered P-pTA-pFol and M_{PEG}-pTA-pFol co-localize in the same cell population, we administered the KB tumor-bearing mice with fluorescently labeled pFol-modified NP pair or their unmodified counterparts and examined the NP distribution on a tissue level (Figure 5). First, P-pTA-pFol or M_{PEG}-pTA-pFol was individually administered to confirm that the two NP signals did not crosstalk (Figure S17–S19). With a mixture of unmodified P and M, their red and green fluorescence signals barely overlapped with a mean Pearson's correlation efficiency of 0.2 (Figure 5 and Figure S20). In contrast, tumors of the animals injected with a mixture of P-pTA-pFol and M_{PEG}-pTA-pFol showed significant overlaps of red and green fluorescence signals with a mean correlation efficiency of 0.9 (Figure 5 and Figure S21), indicating a near-perfect co-localization. These results support that TA helps equalize different NPs and enables their ratiometric delivery to common targets. It is noteworthy that P-pTA-pFol and M_{PEG}-pTA-pFol were distributed in tumor heterogeneously, most likely near the vasculature and at the periphery of tumors due to the binding site barrier effect.^{44, 45} The heterogeneous NP distribution may ultimately limit the benefit of the coordinated delivery enabled by the surface modification. In future studies, pre-treatment with a tumor priming agent may be performed to improve the intratumoral penetration of the NP combinations.

As mentioned earlier, Fe³⁺ was used to achieve efficient and prompt assembly of TA on the surface NPs. The amount of total Fe³⁺ administered to the animals is no more than 8 mg/kg, less than maximum injectable dose of iron (15 mg/kg).^{46, 47} Consistently, animals did not show any signs of abnormality over 24 h observation period after the treatment. Nevertheless, a large dose of Fe³⁺ exceeding the capacity of homeostasis can be toxic⁴⁸; thus, the long-term toxicity of pTA-modified NPs needs to be determined at various dosing regimens. In this regard, other plant-derived polyphenols such as pyrogallol 2-aminoethane, which can efficiently modify surfaces without Fe³⁺,⁴⁹ may be considered a potential alternative to pTA.

In summary, the simple and versatile TA-mediated surface modification equalized the functional properties of NPs with vastly different surface charges or material basis, enabling similar *in vitro* cellular interactions and *in vivo* biodistribution profiles. With this technique, different drugs may be respectively loaded in optimal carriers and camouflaged with a unifying surface layer for coordinated delivery to a common target with a synergistic ratio. With a proper control of drug release kinetics, the colocalization of NPs may translate to codelivery of drug combinations and help achieve a synergistic therapeutic effect.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. PLGA NPs (P) and MSNs (M) modified by pTA and pFol.

(a) TEM and photographic images of suspensions of P, M and the surface-modified forms (scale bar: 100 nm). (b) Relationship between the pFol feed vs. the pFol incorporated in P-pTA-pFol and M_{PEG}-pTA-pFol. Data are expressed as means and standard deviations of 3 identically and independently prepared batches.

Fig. 2. Interactions of individual NPs with KB cells.

(a) Mean fluorescence intensity of KB cells incubated with PS_{COOH} and PS_{NH₂} and the surface-modified forms with and without extra folic acid (1 mM). (b) Mean fluorescence intensity of KB cells incubated with P, P-pTA, P-pTA-Fol, M_{PEG}, and M_{PEG}-pTA-pFol with and without extra folic acid (1 mM). Data are expressed as means and standard deviations of 3 measurements of a representative data set. ****: $p < 0.0001$ by the Sidak's multiple comparisons test. (c) Confocal images of KB cells after incubation with P, M_{PEG} and the surface-modified forms. Scale bars: 10 μ m.

Fig. 3. Interactions of NP mixtures with KB cells.

(a) The extents of PSNH₂-TA-pFol binding to KB cells in the presence of non-fluorescent 125 I-PS_{COOH}, 125 I-PSNH₂-TA-pFol, or 125 I-PS_{COOH}-pTA-pFol as a potential competitor. PSNH₂-TA-pFol vs. 125 I-PS_{COOH}: non-competitive pairs; PSNH₂-TA-pFol vs. 125 I-PSNH₂-TA-pFol and PSNH₂-TA-pFol vs. 125 I-PS_{COOH}-pTA-pFol: competitive pairs. (b) Quantitative measurement of P and M taken up by KB cells after incubation with mixtures of P and M in different ratios. (c) Quantitative measurement of P-pTA-pFol and M_{PEG}-pTA-pFol taken up by KB cells after incubation with mixtures of P-pTA-pFol and M_{PEG}-pTA-pFol in different ratios. (d) Relationship between the feed ratio of P-pTA-pFol to M_{PEG}-pTA-pFol and the measured NP ratio in KB cells after incubation with the mixture. (e) Confocal images of KB cells incubated with mixtures of P and M or P-pTA-pFol and M_{PEG}-pTA-pFol in 1:1 ratio for 2 h. Scale bars: 10 μ m. For a larger field of view of KB cells with P-pTA-pFol + M_{PEG}-pTA-pFol, see Figure S15.

Fig. 4. Quantitative analysis of NPs delivered to KB tumor-bearing mice.

(a) P, M, P-pTA-pFol, and M_{PEG}-pTA-pFol, individually administered (by intravenous injection) and delivered to tumor and liver in 8 h (n=3 mice per NP). (b) P + M or P-pTA-pFol + M_{PEG}-pTA-pFol, co-administered as mixtures (by intravenous injection) and delivered to tumor and liver in 8 h or 24 h (n = 4 mice per NP). *: $p < 0.05$; **: $p < 0.01$ by Sidak's multiple comparisons test following two-way ANOVA.

Fig. 5. Analysis of NP colocalization in KB tumor.

Microscopic images of tumor sections of animals treated with mixtures of P and M or P-pTA-pFol and M_{PEG}-pTA-pFol, sampled at 8 h after intravenous injection and imaged with a confocal microscope. $n = 3$ mice per NP. Two representative images are shown. All acquired images for each animal are shown in Figure S20 and S21. The NP colocalization was analyzed by the Nikon A1R confocal microscope analysis software. The numbers in overlay panels are Pearson's correlation coefficient (R), representing the degree of colocalization: $R=1$ (perfect colocalization), $R=0$ (no colocalization), $R=-1$ (perfect exclusion). 7–10 images per mouse were analyzed. The graph on the right shows pooled data for all animals ($n = 3 \text{ mice} \times 7\text{--}10 \text{ images per mouse} = 21\text{--}30$). ****: $p < 0.0001$ by unpaired t-test.

Table 1.Description and properties^I of NPs

NP	NP description	Z-average (nm, d)	PDI	Zeta potential (mV)	TA content (%)
PS _{COOH}	Polystyrene NPs with carboxyl termini	98.9 ± 4.5	0.12 ± 0.04	-38.6 ± 4.8	Not applicable
PS _{COOH} -pTA	PS _{COOH} coated with pTA	153.7 ± 11.9	0.05 ± 0.01	-38.0 ± 0.8	5.9 ± 0.01
PS _{COOH} -pTA-pFol	PS _{COOH} coated with pFol via pTA	137.0 ± 3.2	0.06 ± 0.01	-20.7 ± 1.4	
PS _{NH2}	Polystyrene NPs with amine termini	106.2 ± 9.7	0.07 ± 0.01	+36.5 ± 3.7	Not applicable
PS _{NH2} -TA	PS _{NH2} coated with TA	134.3 ± 2.7	0.07 ± 0.01	-35.1 ± 0.5	12.6 ± 0.06
PS _{NH2} -TA-pFol	PS _{NH2} coated with pFol via TA	150.3 ± 6.2	0.11 ± 0.02	-18.8 ± 2.4	Not available
Lip	Liposome	159.8 ± 18.1	0.09 ± 0.05	-5.0 ± 1.5	Not applicable
Lip-pTA	Lip coated with pTA	290.5 ± 7.0	0.22 ± 0.07	-30.2 ± 3.5	7.26 ± 0.03
Lip-pTA-pFol	Lip coated with pFol via pTA	221.8 ± 20.3	0.14 ± 0.02	-16.2 ± 2.0	
PLGA	PLGA NP	183.1 ± 2.6	0.07 ± 0.05	-10.6 ± 0.1	Not applicable
P-pTA	P coated with pTA	237.4 ± 10.5	0.10 ± 0.04	-25.8 ± 0.9	7.67 ± 0.35
P-pTA-pFol	P coated with pFol via pTA	213.3 ± 15.6	0.11 ± 0.02	-17.1 ± 0.8	
MSN	Mesoporous silica nanoparticles	231.4 ± 20.9	0.11 ± 0.03	-33.7 ± 1.1	Not applicable
M _{PEG} -pTA	PEG-filled M coated with pTA	235.5 ± 7.6	0.06 ± 0.07	-26.7 ± 0.8	7.7 ± 0.02
M _{PEG} -pTA-pFol	M _{PEG} coated with pFol via pTA	237.2 ± 12.8	0.07 ± 0.01	-17.7 ± 0.5	

NP: Nanoparticles; PS_{COOH}: Polystyrene NPs with carboxyl termini; PS_{NH2}: Polystyrene NPs with amine termini; Lip: liposomes; PLGA: poly(lactic-co-glycolic acid); MSN: mesoporous silica nanoparticles; PEG: polyethylene glycol; M_{PEG}: PEG-filled MSN; TA: tannic acid; pTA: TA-Fe coordination complex; pFol: folate-conjugated and amine-terminated PEG

^INP samples were prepared in bicine buffer (10 mM, pH 7.4), except for M, M_{PEG}-pTA and M_{PEG}-pTA-pFol, which was prepared in phosphate buffer (5 mM, pH 7.4). Z-average and PDI were expressed as means and standard deviations of three batches of NPs. Zeta potentials and TA content are expressed as means and standard deviations of three experiments of same batch of NPs.