Published in final edited form as:

ACS Nano. 2018 October 23; 12(10): 10130–10141. doi:10.1021/acsnano.8b04947.

Dense and Dynamic Polyethylene Glycol Shells Cloak Nanoparticles from Uptake by Liver Endothelial Cells for Long Blood Circulation

Hao Zhou[†], Zhiyuan Fan[†], Peter Y. Li[†], Junjie Deng^{†,‡}, Dimitrios C. Arhontoulis[⊥], Christopher Y. Li[†], Wilbur B. Bowne[§], and Hao Cheng^{*,†,⊥}

[†]Department of Materials Science and Engineering, Drexel University, Philadelphia, Pennsylvania, 19104 USA

[‡]Engineering Research Center of Clinical Functional Materials and Diagnosis & Treatment Devices of Zhejiang Province, Wenzhou Institute of Biomaterials and Engineering, CAS, Wenzhou, 325011 China

[⊥]School of Biomedical Engineering, Science and Health Systems, Drexel University, Philadelphia, Pennsylvania, 19104 USA

[§]Department of Surgery, Drexel University, Philadelphia, Pennsylvania 19102, USA

Abstract

Research into long-circulating nanoparticles has in the past focused on reducing their clearance by macrophages. By engineering a hierarchical polyethylene glycol (PEG) structure on nanoparticle surfaces, we revealed an alternative mechanism to enhance nanoparticle blood circulation. The conjugation of a second PEG layer at a density close to, but lower than the mushroom-to-brush transition regime on conventional PEGylated nanoparticles dramatically prolongs their blood circulation *via* reduced nanoparticle uptake by non-Kupffer cells in the liver, especially liver sinusoidal endothelial cells (LSECs). Our study also disclosed that the dynamic outer PEG layer reduces protein binding affinity to nanoparticles, although not the total number of adsorbed proteins. These effects of the outer PEG layer diminishes in the higher density regime. Therefore, our results suggest that the dynamic topographical structure of nanoparticles is an important factor in governing their fate *in vivo*. Taken together, this study advances our understanding of

* hcheng@drexel.edu.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Additional Tables including pharmacokinetic parameters of PLGA-TPEG-NPs, circulation half-lives of PLGA-TPEG-NPs obtained by fitting the circulation data into two-compartment model via PKSolver, averaged sizes of PLA-TPEG-NPs, and calculated average distance between neighboring chains (*D*) and thickness (*L*) of the outer PEG layer of PLA-TPEG-NPs; addition figures including preparation and size characterization of PLGA-TPEG-NPs, TEM images of PLGA-PEG/TPEG-NPs, characterization of the remaining maleimide on NP surfaces, fluorescence retention of DiD-labeled PLGA-TPEG-NPs, extended blood circulation of PLGA-PEG-NPs from a dynamic outer PEG layer instead of maleimide-thiol linkages, Original ITC data from the study of protein interaction with NPs, IVIS fluorescence image showing a dynamic topographical structure of outer PEG layer reduced NP uptake in the liver, liver cell gating strategy, variation of protein percentage composition in the corona of PLGA-PEG-NPs and PLGA-TPEG-NPs-20, illustration of proposed protein, cell receptor and PEG layer interactions, and NMR characterization of PEG chain density outside NPs.

The authors declare no competing financial interest.

nanoparticle blood circulation and provides a facile approach for generating long circulating nanoparticles.

Graphical Abstract

Keywords

protein corona; nanomedicine; drug delivery; complement activation; grafted polymer; internalization

Nanoparticle (NP)-based therapeutics and imaging agents are promising for many applications. However, successful translation of synthetic NPs to human use has been limited by their short circulation half-lives in the bloodstream. This clearance is believed to be primarily due to opsonin adsorption on NP surfaces, making them visible to cells of the mononuclear phagocyte system.^{1–3} PEG and other polymer grafts on NPs are widely used to reduce protein adsorption, complement activation, and prolong NP circulation.^{2–12} It is generally accepted that a thick and dense PEG layer is necessary for this purpose as dense chains prevent protein adsorption by steric repulsion.^{13–16} Furthermore, polydisperse PEG brushes of varying length have been shown to be more effective than monodisperse PEG layers in reducing protein adsorption,^{17, 18} indicating the design may generate long circulating NPs.¹⁹ The improvement was attributed to the increased PEG density and layer thickness because short PEG chains fit in between longer PEG molecules, and steric repulsions between grafted chains increase the overall thickness of the PEG layer. However, some studies have reported the existence of an optimal density of grafted PEG for repelling proteins.^{20–22} One explanation from theoretical analysis is that the PEG layer transitions from a mushroom regime to a brush regime as the polymer grafting density increases.²³ A mushroom regime refers to a low PEG density, in which the dynamic chain coils rarely interact laterally on surfaces, while a brush regime is a higher density condition where chains are confined and extended from substrates.²⁴ The thermally agitated conformational fluctuation of low-density PEG chains increases the characteristic adsorption time and therefore kinetically slows down protein adsorption, while confined chains at a high PEG grafting density do not have this effect.²³ Therefore, there is a fundamental design trade-off between steric repulsion and conformational fluctuation. Understanding and maximizing both effects would substantially advance polymer graft design for enhanced NP circulation.

Here, we report the generation of a PEG cloak with dual physical effects for NPs (Figure 1a). This design was based on the hypothesis that NPs with a dense inner (primary) PEG

layer would thermodynamically prevent proteins from accessing NPs *via* steric repulsion,^{25, 26} while an outer layer of PEG in the mushroom regime would provide a topographical structure for NPs for harnessing the conformational fluctuation effect to kinetically interfere with protein binding, leading to long NP circulation. Although NP physicochemical properties such as shape, size, elasticity, surface chemical composition, crystallization, and stability have been investigated for extending NP circulation,^{15, 27–32} the impact of NP dynamic topographical structures have yet to be elucidated. Indeed, we found that an outer layer of PEG at a density close to the mushroom-to-brush transition regime creates an ideal topographical structure to enhance blood circulation and tumor accumulation of conventional PEGylated NPs. Surprisingly, the dramatically extended NP circulation time stems from reduced NP uptake by liver sinusoidal endothelial cells (LSECs) rather than macrophages, which have been the focus of previous studies of generating long circulating NPs.^{2, 15, 33–35} In addition to providing a different mechanism for generating long circulating NPs and revealing the dynamic topographical structure as a factor for consideration in NP design, this study helps to fundamentally understand some previously reported results of PEGylated NPs.

RESULTS AND DISCUSSION

Dynamic topographical structure of PEG shells prolongs the circulation of PEGylated NPs.

Widely used poly(lactic-co-glycolic acid)-*b*-poly(ethylene glycol) NPs (PLGA-PEG-NPs) were first selected for this study. To create PLGA-PEG-NPs with controlled topographical structures of PEG shell (PLGA-TPEG-NPs), methoxy PLGA_{20K}-PEG_{5K} was mixed with maleimide-terminated PLGA_{20K}-PEG_{5K} (PLGA-PEG-MAL) and formed maleimide-functionalized PLGA-PEG-NPs *via* nanoprecipitation.³⁶ The resulting particles were then coupled with methoxy-PEG_{2K}-thiol *via* the thiol-maleimide reaction to generate a hierarchical PEG shell (Figure 1a). The polydispersity index of PEG molecules or blocks was approximately 1.05. To study the effect of topographical structure on NP blood circulation, PLGA-TPEG-NPs with a series of PLGA-PEG-MAL percentages were prepared (PLGA-TPEG-NPs-10, 20, 40, 60, 80, and 100, in which the numbers indicate the percentages of PLGA-PEG-MAL in NP fabrication). NPs with different outer layer PEG densities were fabricated to have similar average sizes between 85–97 nm and similar zeta potentials at –2 mV (Figure S1). The average size of PLGA-TPEG-NPs-20 measured with dynamic light scattering (DLS) was approximately 90 nm (Figure S1b), while it was around 50 nm (48.6 ± 8.9 (SD) nm) in the transmission electron microscopy (TEM) image of the NPs (Figure 1b) similar to those of PLGA-PEG-NPs and PLGA-TPEG-NPs-100 (Figure S2). Further characterization showed that the remaining maleimide on the NP surfaces was undetectable (<1%), suggesting a complete reaction (>99%) during the conjugation step (Figure S3).

The circulation of 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate (DiD)-labeled NPs in the blood were evaluated in BALB/c mice based on the fluorescence signal of DiD in the plasma (Figure 1c). DiD labelling is well-accepted in NP circulation studies because of the slow release of DiD from NPs,³³ as confirmed by us that around 7% of DiD was released from NPs *in vitro* after 48 h (Figure S4). The circulation data were analyzed to

calculate circulation half-lives (Figure 1d) and other pharmacokinetic parameters (Table S1) by applying a pharmacokinetic one-compartment model *via* PKSolver.³⁷ PLGA-TPEG-NPs-20 showed the longest circulating half-life of 10.01 ± 0.52 h, which was approximately three times of the 3.48 ± 0.36 h circulation half-life of conventional PLGA-PEG-NPs made of 100% methoxy-PLGA_{20K}-PEG_{5K}. The circulation half-life of NPs started to decrease when the density of the outer PEG layer further increased. PLGA-TPEG-NPs made of 80% and 100% PLGA-PEG-MAL showed similar circulation half-lives to the conventional PLGA-PEG-NPs, even though they possess longer elimination half-lives when calculated using a two-compartment model (Table S2).

To exclude the effect of the maleimide-thiol linkage on NP circulation, methoxy-PEG-thiol with two ethylene glycol units was used to quench the maleimide groups. The resulting PLGA-PEG-NP-20 has the same amount of maleimide-thiol linkage as PLGA-TPEG-NP-20, but without the outer PEG layer. Its blood circulation profile was similar to that of conventional PLGA-PEG-NPs (Figure S5), indicating the prolonged circulation of PLGA-TPEG-NP-20 results from the outer PEG layer. This two-step NP fabrication method is convenient to functionalize NPs with biomolecules on the primary PEG layer instead of the outer most layer to minimize the conjugated biomolecule-induced NP clearance.³⁸ It is known that a fraction of PEG chains may entrap inside NP hydrophobic cores such as PLGA during nanoprecipitation.³⁹ The two-step NP fabrication allows us to quantify PEG chains in the outer PEG layer for determining their conformation.

Outer PEG layer close to the mushroom-to-brush transition regime is optimal for NP circulation.

To understand the topographical chain conformation that affects NP blood circulation, the chain densities in the outer PEG layer were evaluated using FITC-PEG_{2K}-thiol in the conjugation step and measuring the FITC fluorescence intensity of NP solutions.⁴⁰ The chain densities can be obtained after knowing FITC-PEG_{2K} concentration, NP concentration, and NP size. The distance between neighboring chains (D) was calculated using

$$D \approx A^{1/2},$$

in which A is the average area occupied by one PEG chain. The Flory radius (end-to-end distance) of an unperturbed PEG coil in the outer layer is calculated using^{24, 41}

$$R_F \approx N^{3/5} a,$$

in which N is the degree of polymerization ($N = 45$ for PEG_{2K}), and a is the monomer size, ($a = 0.35$ nm for PEG).⁴⁰ In the outer layer, the thickness in the mushroom regime ($D > R_F$) and mushroom-to-brush transition regime is simply equal to or close to R_F , while in the brush regime ($D \ll R_F$), the thickness can be estimated using²⁴

$$L \approx Na^{5/3} D^{-2/3}.$$

Experimental study of grafted PEG_{2K} has shown that the layer thickness in the mushroom regime is R_F , matching well with the theoretical estimation. It was also found that the PEG layer thickness started to increase when D was reduced to around R_F ($A \approx R_F^2$),^{42, 43} reaching the standard mushroom-to-brush transition regime.^{24, 41}

The hydrated NP sizes were first used in the estimation of NP concentrations, surface areas, and PEG densities in the outer layers.^{44, 45} This standard method in the field showed the maximum circulation time occurs when the outer layer PEG density is at $D \approx R_F$ (Figure 1f). However, the standard method underestimated NP concentrations in solutions because hydrated NPs include the weight of water molecules. When the dehydrated NP size from TEM (50 nm) was used to calculate NP concentrations, and hydrated NP sizes were used to calculate surface areas, the optimal PEG density in outer layer was at $D \approx 2R_F$ in which the mushrooms are close to each other, but have no appreciable lateral interactions. The optimal outer layer PEG density is reasonable to be slightly lower than the mushroom-to-brush transition regime. This is because the lateral interaction among neighboring PEG chains at $D \approx R_F$ can significantly reduce chain compressibility,⁴² limiting chain fluctuation. Considering the diameters of plasma proteins are mostly around or larger than R_F (3.5 nm), PEG chains at a density $D \approx 2R_F$, which allows free chain fluctuation, may be sufficient to protect NP surfaces *via* interfering with protein binding.

Dynamic outer PEG layer reduces protein binding affinity on PEGylated NPs.

One of the major factors in determining NP fate *in vivo* is their protein corona, which is formed *via* protein adsorption on NPs in the blood.^{46, 47} To understand whether the outer PEG layer at the optimal density that gives the longest circulating NPs affect protein adsorption, NP-protein interactions were analyzed using isothermal titration calorimetry (ITC), which can directly measure protein adsorption to NPs in physiological media.⁴⁸ Saline with 20% fetal bovine serum (FBS) was titrated into the NP solutions, and the change in heat for every titration was measured (Figure S6) and integrated (Figure 2a-d). The resulting integrated heat was fitted into a one-site binding model where the protein adsorption enthalpy (ΔH), stoichiometry (Na , which is the average number of proteins adsorbed on a single NP), and binding constant (Ka) were calculated (Figure 2e,f).⁴⁰ Since whole serum proteins exist in the system, the detected Na and Ka are apparent numbers, reflecting the “average” interaction between NPs and proteins.

The adsorption of serum proteins on the surfaces of PLGA-NP, PLGA-PEG-NPs, PLGA-TPEG-NPs-20, and PLGA-TPEG-NPs-100 are all exothermic. The results from PLGA-NPs and PLGA-PEG-NPs are in agreement with previous studies that PEGylation reduces protein adsorption.^{40, 44} Interestingly, PLGA-PEG-NPs, PLGA-TPEG-NPs-20, and PLGA-TPEG-NPs-100 showed similar numbers of adsorbed proteins on NPs (Na). This is likely because the NPs have a similar particle size and PEG density in the primary PEG layer, resulting in similar numbers of protein binding sites. Some studies have shown that PEG density is the most important factor in determining the amount of adsorbed proteins on

substrates, while variation of PEG length has a limited effect.⁴⁹ PLGA-TPEG-NPs-100 can be simply considered as PLGA-PEG-NPs with a longer PEG length. However, the binding affinity of proteins to NPs with a hierarchical PEG shell (PLGA-TPEG-NPs-20) was significantly less than those of the conventional PLGA-PEG-NPs and PLGA-TPEG-NP-100. This dynamic effect of the outer PEG layer may affect the adsorption of proteins that mediate NP clearance directly or indirectly as proteins interact with those that are already adsorbed on NPs. This dynamic effect is also expected to be more dramatic for large sized proteins because they have more chances to interact with fluctuating PEG chains than their smaller sized counterparts.

Dynamic outer PEG layer reduces the clearance of PEGylated NPs by non-Kupffer cells in the liver.

To understand how the dynamic outer PEG layer affect NP clearance in the blood circulation, NP uptake by cells was studied *in vivo*. The 4 h time point after systemic NP administration was selected for the study because there were approximately 60% of conventional PLGA-PEG-NPs and 30% of PLGA-TPEG-NPs-20 cleared at the time (Figure 1c), giving a significant difference for comparison. According to the IVIS image of tissues collected at this time point (Figure S7), NPs were mainly cleared by the liver and spleen. PLGA-TPEG-NPs-20 showed a similar biodistribution as conventional PLGA-PEG-NPs in most tissues except a significantly lower uptake in the livers. Therefore, the *in vivo* study of NP cellular uptake was focused on liver cells.

For the study, the livers were perfused with a collagenase solution *in situ*, and the isolated cells were analyzed with flow cytometry. It was found in the histograms of total isolated cells that liver cells from the mice treated with PLGA-PEG-NPs showed a single peak (A) at a position of high DiD (NP fluorescence) intensity, while liver cells from PLGA-TPEG-NP-20-treated mice showed two peaks at positions of intermediate and high DiD intensities (B & C) (Figure 3a). The peak split and shift demonstrate that the dynamic outer PEG layer reduces the uptake of PEGylated NPs in some liver cells.

To identify the cells, the isolated cells were classified into four groups according to marker staining and forward and side scatter of light: hepatocytes (large CD146⁺CD68⁻ cells), LSECs (CD146⁺CD68⁻ cells), Kupffer cells (CD146⁻CD68⁺ cells), and other cells (small CD146⁻CD68⁻ cells) including hepatic stellate cells, B cells, and others (Figure S8). Hepatocytes, LSECs and Kupffer cells are the most abundant cells in livers and were reported to account for 57%, 23% and 15% of total liver cells, respectively.⁵⁰ The analysis of DiD⁺ cells shows that the majority of LSECs and Kupffer cells internalized the NPs. However, only a small fractions of hepatocytes and other liver cells internalized PLGA-PEG-NPs, and the percentages were even lower for PLGA-TPEG-NPs-20 (Figure 3b). Further analysis of DiD intensity reveals that the dynamic outer PEG layers dramatically reduced the internalization of PEGylated NPs by LSECs as shown by the shift of DiD peak from A to B (Figure 3c), but slightly increased NP uptake in Kupffer cells with DiD peak shifted from A to C (Figure 3d). The hepatocytes and other liver cells that internalized a high amount of NPs almost disappeared when PEGylated NPs carry an outer PEG shell (Figure 3e,f). The quantitative result (Figure 3g) indicates that LSECs can be equally as important as

Kupffer cells in clearing some types of NPs, and the reduced NP uptake by LSECs is the major reason why dynamic topographical PEG layer extended NP blood circulation.

These results are somewhat surprising because macrophages like Kupffer cells are normally considered as the major scavengers of NPs in the blood.^{2, 15, 33–35} Although LSECs express scavenger receptors, have a high endocytic capacity beyond the complement-mediated internalization,⁵¹ and can uptake NPs,^{51–54} it was largely unknown that reducing NP internalization by LSECs, but not macrophages can be sufficient to dramatically extend NP blood circulation. The slight increase of PLGA-TPEG-NPs-20 in Kupffer cells may be because more PLGA-TPEG-NPs-20 than PLGA-PEG-NPs are locally available to Kupffer cells, which spread on top of LSECs in sinusoids, when LSECs are not efficient in removing PLGA-TPEG-NPs-20.

There were less than 6% of hepatocytes and other cells internalized NPs, these cells may belong to the cell layers next to sinusoidal blood vessels. The dynamic outer PEG layer also significantly reduced NP internalization in these subgroups of hepatocytes and other cells as quantified by the relative MFI of the cells that internalized NPs (DiD⁺) (Figure 3h,i).

As an attempt to understand the results, we employed liquid chromatography–mass spectrometry (LC-MS) to identify and compare the most abundant proteins adsorbed on PLGA-PEG-NPs and PLGA-TPEG-NPs-20 *in vitro* in FBS. It was found the outer PEG layer altered the composition of these proteins in the corona at different levels (Figure S9). It remains to be determined whether the variation of specific proteins result in the difference of NP uptake in the liver. According to recent publications, apolipoprotein and the natural ligands of the scavenger receptor, stabilin-2 are worth special attention.^{6, 35, 54}

On the other hand, the decrease in protein binding affinity to NPs by the outer PEG layer (Figure 2f) reflects the alteration of protein binding kinetics. The reduced PLGA-TPEG-NPs-20 uptake by LSECs may also be because the conformational fluctuation of low-density PEG chains in the outer layer increases the characteristic binding time (the time required to establish a physical bond) between LSEC receptors and their plasma protein ligands adsorbed on NPs. Therefore, it kinetically slows down PLGA-TPEG-NPs-20 uptake by the cells (Figure S10a). This explanation is supported by our previous study, in which an enzyme was conjugated on the primary PEG layer of PLGA-PEG-NPs.³⁸ The addition of an outer PEG layer decreased the apparent enzyme activity possibly by interfering with the enzyme to access its substrate. The outer PEG layer on NPs did not reduce their uptake by Kupffer cells possibly because Kupffer cells clear NPs mainly through the recognition of complement proteins on NPs.⁵⁵ Complement proteins covalently bind to the proteins that are intercalated into grafted polymers.² The extension of complement proteins, which have high molecular weights, away from NPs makes their access by Kupffer cells unaffected by the outer layer chain fluctuation (Figure S10b).

NP clearance depends on hydrodynamic shears as cells lose the necessary time to capture NPs under a high flow velocity.¹ The hydrodynamic effect becomes critically important if PEG outer layers interfere with NP binding to LSEC receptors because more time would be needed to establish the bonds compared with conventional PEGylated NPs. Hydrodynamic

force has also been shown to strongly affect the relaxation of worm-like micelles in flow. Long cylindrical NPs are stretched out along the flow direction and pulled away from phagocytes by hydrodynamic shears, reducing their internalization by the cells.²⁷ These two hydrodynamic effects are somewhat different and may be synergized to generate ultra-long circulating NPs by controlling both NP shape and dynamic topographical structure.

Further validation of dynamic hierarchical PEG shells in generating long circulating NPs.

To further demonstrate the prolonged blood circulation of PEGylated NPs by a dynamic outer PEG layer, poly(lactic acid)-poly(ethylene glycol) NPs (PLA-PEG-NPs) and PLA-PEG-NPs with controlled topographical structures of PEG shells (PLA-TPEG-NPs) were investigated. PLA-PEG-NPs and PLA-TPEG-NPs were prepared *via* the aforementioned method, but using methoxy PLA_{16K}-PEG_{5K} and PLA_{16K}-PEG_{5K}-MAL. The size of PLA-TPEG-NPs was around 85 nm as measured by DLS (Table S3), similar to those of PLGA-TPEG-NPs.

It was found that the blood circulation half-life of PLA-PEG-NPs from the one-compartment model is 13.6 ± 0.7 h, which is dramatically longer than that of PLGA-PEG-NPs. In addition to the chemical structure difference between PLGA and PLA, another possible reason behind the result was the high PEG density, $0.47/\text{nm}^2$ on PLA-PEG-NPs, while the density was $0.38/\text{nm}^2$ for PLGA-PEG-NPs as measured by ¹H NMR (Figure S11). These numbers are close to the previously reported PEG densities on PLA-PEG-NPs and PLGA-PEG-NPs.^{44, 45} PLA-TPEG-NPs prepared with 5, 10 and 20% of PLA-PEG-MAL were studied for blood circulation. The dynamic topographical structure of PEG shells significantly extended NP blood circulation to 22.2 ± 1.7 h for PLA-TPEG-NPs-10 (Figure 4). Interestingly, the optimal PEG density in the outer layer was similar to the case of PLGA-TPEG-NPs (Table S4).

The blood circulation half-life of PLA-TPEG-NPs-20 was only slightly higher than that of PLA-TPEG-NPs-5 (Figure 4a,b). Similarly, the circulation times of PLGA-TPEG-NPs-10 and PLGA-TPEG-NPs-40 were in close proximity (Figure 1c,d). These results may be explained by the effective fluctuation of PEG chains. Although sparse PEG chains in the outer layer can fluctuate freely to interfere with proteins or cell receptors binding to NPs, the number of chains is insufficient to cover the entire NP surface (Figure 1f). On the other hand, interactions among neighboring PEG restrain their fluctuation and ability to interfere with protein binding when the PEG density in the outer layer is significantly higher than the optimal density or even in the brush regime. A previous study of PEGylated hydrogel NPs also found no significant difference in NP blood circulation when PEG layers were in a mushroom regime and a brush regime.⁴⁰ It is worth noting that the PEG densities in the previous study were close to the densities of the outer PEG layer in our study, but a few times lower than the density of our primary PEG layer. In our case, the dense primary PEG layer transforms the hydrophobic PLGA and PLA cores into hydrophilic NPs, resembling the hydrogel NP cores in the previous study. The grafted PEG layers of the hydrogel NPs function similarly as the outer PEG layers of PLGA-TPEG-NPs and PLA-TPEG-NPs in extending NP blood circulation. An optimal density might exist in between their studied mushroom and brush regimes. Chain fluctuation can also explain why a low-density PEG

shell is sufficient to dramatically extend the circulation of liposomes.^{56, 57} Therefore, our study provides a panoramic view to understand the previous NP blood circulation results. It also demonstrates the importance of a high density PEG layer in extending the circulation of hydrophobic NPs, and there is an optimal PEG density to establish a dynamic topographical structure. When combined together, the PEG shell with dual physical effects is highly efficient in generating long circulating NPs as approximately 20% of PLGA-TPEG-NPs and 48% of PLA-TPEG-NPs remained in the blood 24 h post injection. Compared to coating NPs with cell membranes, an emerging method to increase NP blood circulation, our facile and inexpensive strategy generates NPs with a longer circulation half-life in the one-compartment model, although cell membrane-coated NPs possess a longer elimination half-life.^{33, 58, 59}

Prolonged NP blood circulation increases their tumor accumulation.

One promising application of long-circulating NPs is cancer therapy. A long circulation half-life is important for NPs to efficiently accumulate in solid tumors through the enhanced permeability and retention (EPR) effect and/or other mechanisms.^{38, 60, 61} To study the effect of a dynamic topographical structure of PEG shells in NP tumor accumulation, BALB/c mice bearing 4T1 tumors were used to study the biodistribution of DiD-labeled NPs. The biodistribution of PLGA-PEG-NPs, PLGA-TPEG-NPs-20, PLA-PEG-NPs, and PLA-TPEG-NPs-10 were studied 48 h after tail vein injection of the NPs. The result shows NP tumor accumulation is correlated to their blood circulation (Figure 5). Quantitative analysis was performed by measuring the DiD signal of homogenized tissues (Figure 5c). Extended NP circulation increased PLGA-PEG-NP accumulation in tumors from 2.4%ID/g (4.2%ID) to 6.7%ID/g (7.8%ID) at 48 h post NP injection, while the improvement for PLA-PEG-NPs was from 6.7%ID/g (7.5%ID) to 11.5%ID/g (11.4%ID). Compared to the less than 1%ID NP tumor accumulation in many studies,⁶² these high accumulation efficiencies may also result from a strong EPR effect of 4T1 tumors in addition to the long blood circulation of NPs.

CONCLUSIONS

In summary, we have demonstrated that a dynamic topographical structure of PEG shells dramatically prolongs the blood circulation time of conventional PEGylated NPs by reducing NP uptake by LSECs instead of macrophages. Our study indicates that it may be necessary to reduce the uptake of NPs by both macrophages and LSECs for generating ultra-long circulating NPs. The optimal density of the outer PEG layer in the hierarchical PEG shell is close to the mushroom-to-brush transition regime, in which the fluctuating PEG segments reduce protein binding affinity with NPs, but not the total amount of adsorbed proteins. The mechanism is beyond the commonly known steric effect and hydrophilicity of PEGs in resisting protein adsorption. The dynamic effect disappears when PEG chains in the outer layer are in a high density regime. In addition to the topographical structure determined by the outer PEG layer, a high density primary PEG layer is also important for generating long circulating hydrophobic NPs. Our facile approach to generate long circulating NPs may have broad applications including cancer therapy and diagnosis.

METHODS

Materials.

Methoxy poly(ethylene glycol)-*b*-poly(lactic-co-glycolic acid) (Mw ~5000:20000 Da) (PLGA-PEG), poly(lactic-co-glycolic)-*b*-poly(ethylene glycol)-maleimide (Mw ~20000:5000 Da) (PLGA-PEG-MAL), methoxy poly(ethylene glycol)-*b*-poly((D,L) lactic acid) (MW ~5000:16000 Da) (PLA-PEG) and maleimide-poly(ethylene glycol)-*b*-poly(D,L-lactide) (MW ~5000:16000 Da) were purchased from Polysciotech Inc. Methoxy-poly(ethylene-glycol)-thiol (Mw~2000 Da and Mw~5000 Da) (PEG-SH) were purchased from Laysan Bio Inc, while FITC-Poly(ethylene glycol)-thiol (Mw~2000 Da) was obtained from Nanocs. Mouse breast cancer cell line (4T1) was purchased from ATCC Inc. DiD oil (DiI18(5) oil) was a product of Life Technologies. Maleimide quantification assay was a product of Abcam®. Additional salts, solvents and buffers were purchased from Fisher Scientific. Antibodies for flow cytometry analysis were all purchased from Biolegend.

Fabrication of NPs with controlled topographical structures.

PLGA-TPEG-NPs and PLA-TPEG-NPs were prepared according to a nanoprecipitation method previously reported.^{36, 38} PLGA_{20K}-PEG_{5K}/PLGA_{20K}-PEG_{5K}-maleimide (PLGA_{20K}-PEG_{5K}-MAL) or PLA_{16K}-PEG_{5K}/PLA_{16K}-PEG_{5K}-maleimide (PLA_{16K}-PEG_{5K}-MAL) were dissolved in acetonitrile at the indicated weight ratio and concentration. A fluorescent dye, 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate (DiD), was also dissolved at a weight ratio of 0.1% to polymers. The acetonitrile solution of polymers and DiD was added dropwise into PBS (2:5 v/v) under stirring. After 2 h, the solution was kept overnight under vacuum to evaporate the organic solvent. PEG_{2K}-thiol (PEG_{2K}-SH) or PEG_{5K}-thiol were added to the NP solution to build the outer PEG layer on NP surfaces. The final concentration of PEG_{2K}-SH was 2 mg/mL. The obtained PLGA-TPEG-NPs or PLA-TPEG-NPs were purified *via* overnight dialysis using a home-made dialysis cassette with 50 nm membranes (Whatman®) against saline. The homemade dialysis cassettes were used for all the NP dialysis in this study. NPs were concentrated to the desired concentrations indicated by fluorescence intensity through centrifugal filters (Amicon Ultracel 100K) and passed through 0.2 µm syringe filters before further use.

Characterization of NPs.

Dynamic light scattering (Zetasizer Nano ZS90, Malvern) was used to detect NP sizes. Transmission electron microscopy (TEM) was used to image particles. About 10 µL of 0.2 mg/mL NPs was added to the carbon coated grid pre-cleaned by plasma. Then, 5 µL of 2% uranyl acetate in water was dropped on the grid, which was rinsed with DI water three times. The solution was removed instantly with a filter paper, and this staining step was repeated three times. The sample was imaged *via* JEOL JEM2100 at 200 kV. To quantify NP size from TEM images, 45 NPs from 3 TEM images were measured.

Maleimide quantification assay.

To quantify the amount of maleimide on NP surfaces before and after reacting with PEG_{2K}-SH, a fluorimetric assay (Abeam, ab112141, Cambridge, MA) was used according to the

manufacturer's instructions. Briefly, MalemGreen Indicator has enhanced fluorescence upon reacting with a maleimide. Fluorescence intensity was read at 490/520 nm (Ex/Em) in a 96-well plate after mixing MalemGreen Indicator (50 μ L) with test samples (50 μ L) of NP solution (1 $\times$), its 10 fold (0.1 $\times$) and 100 fold (0.01 $\times$) dilutions before reaction as well as NP solution (1 $\times$) after reaction. Concentrations for 1 $\times$ solution of PLGA-PEG-NPs composed of 20%, 80%, and 100% PLGA-PEG-MAL in preparation were 50, 20, and 20 mg/mL respectively to fit maleimide concentrations into the detection sensitive range. All measurements were made in triplicate.

***In vivo* circulation study.**

DiD-encapsulated NPs at 10 mg/mL in saline were systemically administered through tail vein injection into female BALB/c mice (Charles River Laboratory). Each mouse received 100 μ L of NP solution. About 15 μ L of blood was collected at 2 min, 15 min, 0.5 h, 1 h, 2 h, 4 h, 8 h, 24 h, and 48 h post i.v. injection. The blood was diluted in 200 μ L of PBS containing 16 U/mL heparin as an anti-coagulant. Blood cells were removed by spinning at 300 g for 5 min, and 180 μ L of the supernatant was used for testing fluorescence intensity using an Infinite 200 microplate reader (TECAN) (excitation/emission: 600/665 nm). *In vivo* blood circulation half-lives were calculated based on the one-compartment model of pharmacokinetics *via* PKSolver. All animal procedures were conducted in accordance to the protocols approved by the Drexel Institutional Animal Care and Use Committee in compliance with NIH guidelines.

Quantification of PEG density in the outer layer on NPs.

FITC-PEG_{2K}-SH instead of PEG_{2K}-SH was conjugated on NP surfaces *via* thiol-maleimide reaction. After 3 h stirring at room temperature, NPs were dialyzed against PBS overnight to remove unreacted FITC-PEG_{2K}-SH. The dialysis buffer was changed 3 times until fluorescence intensity in the buffer was undetectable. After dialysis, the total number concentration of PEG chains in the outer PEG layer ($C_{outer\ PEG}$) was quantified by comparing FITC fluorescence intensity of the collected NP solution with a FITC-PEG_{2K}-SH standard curve. The weight concentration of the collected NPs (W_{NP}) was quantified by comparing the fluorescence intensities of the encapsulated DiD before and after dialysis. Based on W_{NP} , the number concentration of NPs and total NP surface area were calculated using two methods. One standard method in the field involves the use of average NP size measured from DLS (d_{DLS}) and a hydrated NP density (ρ) of 1.2 g/cm³ to calculate the average volume and weight of each NP.^{44, 45} The average area occupied by each PEG chain in the outer PEG layer (A) was calculated as

$$A = \frac{W_{NP} \times 4\pi \left(\frac{d_{DLS}}{2}\right)^2}{\frac{4}{3}\pi \left(\frac{d_{DLS}}{2}\right)^3 \times \rho \times C_{outer\ PEG}}.$$

The other method uses the average NP size (50 nm) measured from TEM and the density of PLGA, 1.34 g/cm³, to calculate the average volume and weight of each NP. In the estimation

of hydrated NP surface area, d_{DLS} minus the outer layer mushroom thickness ($L=3.5$ nm) was used. Then A was calculated as

$$A = \frac{W_{NP} \times 4\pi \left(\frac{d_{DLS}}{2} - L \right)^2}{\frac{4}{3}\pi \left(\frac{d_{TEM}}{2} \right)^3 \times \rho \times C_{outer\ PEG}}.$$

ITC study on protein-NP interaction.

DiD-encapsulated NPs were prepared *via* the method mentioned above and were concentrated to 5 mg/mL based on DiD fluorescent intensity. For PLGA-NPs without PEG, 50:50 Poly(DL-lactide-co-glycolide) (PLGA, 0.66 dL/g) was dissolved in acetone at 5 mg/mL, and PLGA-NPs were prepared by adding 1 mL of the polymer solution into 5 mL saline. All the solutions were dialyzed against saline overnight. The ITC tests were performed in a VP-ITC microcalorimeter (MicroCal, LLC) at 37 °C. Saline with 20% FBS was injected into the sample cell containing NP solutions with a stirring speed of 300 rpm. PLGA-NPs, PLGA-PEG-NPs, PLGA-TPEG-NPs-20 and PLGA-TPEG-NPs-100 were detected at concentrations of 2, 5, 5, and 5 mg/mL, respectively. The molar concentration of FBS is estimated by the total protein concentration of 38 g/L and an averaged protein molecular weight of 66 KDa, since the majority of the proteins are albumin (23 g/L).⁶³ The molar concentration of NPs is estimated based on the particle size measured *via* DLS and a density of 1.2 mg/mL. Titration of FBS was performed as a first injection of 2 µL, followed by 20 injections of 5 µL and 15 injections of 10 µL with a spacing of 300 s and a reference power of 5 µcal/s. The ITC measurements were analyzed and fitted into a one-site binding model in Origin Software. The background of dilution enthalpy was subtracted from the plots.

Liver cell isolation.

PLGA-PEG-NP or PLGA-TPEG-NP-20 were injected i.v. *via* tail vein. After 4 h, mice were sacrificed *via* carbon dioxide asphyxiation and immediately perfused with 15 mL warm Hank's balanced salt solution (HBSS) with 0.5 mM EDTA *via* the portal vein. An incision was made in the inferior vena cava to drain out blood and solution from the liver. Next, the perfusion solution was switched to 15 mL of warm digestion medium consisting of DMEM (Coming) with 100 U/mL collagenase IV (Worthington) and the liver was slowly perfused for 5-7 min. The liver was then excised and placed in 5 mL of the digestion medium. To release the cells, the liver was mechanically separated using forceps in a petri dish containing digestion medium and incubated at 37 °C for 20 min. The released cells were separated from liver stroma by filtering through sterile gauze. The cells were spun at 400 g for 5 min, red blood cells were removed using red blood cell lysis buffer (Biolegend), and then washed twice in DMEM.

Flow cytometry analysis of NP internalization by liver cells *in vivo*.

Single cell suspension collected from liver fractionation is stained with fluorophore-conjugated antibodies for markers: anti-CD146-PerCP/Cy5.5 and anti-CD68-PE/Cy7

(BioLegend). Cells were first stained with anti-CD146 for 30 mins at 4 °C and was then washed. Intracellular staining of CD68 was processed after washing, fixing and permeabilizing the CD146-stained cells. 150,000 events were collected for each sample with a BD FACSCanto (BD Bioscience) and the result was analyzed with FlowJo (Tree Star, Inc). Compensation was calculated using single-color stained samples. Debris was excluded based on forward scattering measurement (FSC-A) and singlets were gated with similar area and height in forward scattering measurement (FSC-A vs. FSC-H). Hepatocytes, endothelial cells, Kupffer cells and other cells are gated according to their sizes and markers.

Proteomic identification of NP-binding proteins.

DiD-labeled PLGA-PEG-NPs (0.6 mL, 20 mg/mL in saline) and PLGA-TPEG-NPs-20 (0.6 mL, 20 mg/mL in saline) were incubated with 0.6 mL of FBS for 30 min at 37 °C under shaking at 100 rpm. NPs were spun down at $20,000 \times g$ at 4 °C for 1 h and the supernatant was removed. NP pellets were resuspended in 2 mL Dulbecco's phosphate buffered saline (DPBS) with calcium and magnesium (Corning, Cellgro) and washed twice under the same centrifugation conditions. Afterwards, 20 μ L of 2% sodium dodecyl sulfate (SDS, Sigma) in PBS was added to resuspend NPs. The solutions were incubated at 4 °C overnight to elute proteins associated with NPs. NP solutions were centrifuged at $20,000 \times g$ at 4 °C for 1 h and supernatants were collected for protein analysis. To remove residual NPs and aggregates, the protein samples were run into NuPage 10% Bis-Tris Gels. The gel regions with proteins were cut and digested with trypsin. The digests were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) on a Q Exactive HF mass spectrometer. MS/MS spectra generated from the LC-MS/MS runs were searched using full tryptic specificity against the UniProt bovine database (www.uniprot.org) using the MaxQuant 1.6.1.0 program. Protein quantification was performed using unique+razor peptides (nonredundant peptides). Razor peptides are shared (non-unique) peptides assigned to the protein group with the most other peptides (Occam's razor principle). False discovery rates for protein and peptide identifications were set at 1%. After identifying the most abundant 30 proteins in the protein corona of PLGA-PEG-NPs, the percentage composition of these proteins for PLGA-TPEG-NPs-20 and PLGA-PEG-NPs were compared.

NMR quantification of PEG chains on NPs.

To quantify the densities of primary PEG layers on PLGA-PEG-NPs and PLA-PEG-NPs, the NPs were prepared in deuterium oxide (D_2O). 0.2 mL of polymer in acetonitrile was added dropwise into 0.5 mL D_2O in a small vessel to minimize the water vapor. In sample preparation, 5 mg of sodium benzenesulfonate was also dissolved in D_2O as an external reference.⁶⁴ 1H NMR spectra of NPs in D_2O were detected *via* 300MHz Varian at room temperature. The 1H NMR spectra show that the protons of PEG units and sodium benzenesulfonate are 3.75 ppm and 7.6-7.7 ppm, respectively (Figure S11a,b). By assuming that PEG chains entrapped inside the hydrophobic core of NPs cannot be detected,⁶⁵ the amount of PEGs on the NP surfaces can be measured by comparing the integral ratio of $I_{3.75 \text{ ppm}} : I_{7.6-7.7 \text{ ppm}}$. Taking into account the total amount of polymers in the NMR samples, it was found that approximately 85% of PEGs were on PLGA-PEG-NP surfaces, while almost 100% of PEGs were on PLA-PEG-NP surfaces.

Quantification of PEG density in the primary layer on NPs.

To be consistent with the existing publications for comparison,^{44, 45} the hydrated NP size measured from DLS was used for the calculation. The PEG densities in the primary layer of the PLGA-PEG-NPs and PLA-PEG-NPs were calculated as:

$$\frac{1}{A} = \frac{M_{PEG} \times N_A}{MW_{PEG}} \times \frac{\rho \times \frac{4}{3} \pi \left(\frac{d_{DLS}}{2}\right)^3}{M_{polymer} \times 4\pi \left(\frac{d_{DLS}}{2}\right)^2},$$

in which M_{PEG} is the weight of PEGs outside NPs measured *via* NMR signals, N_A is Avogadro's number, MW_{PEG} is 5,000 g/mole, and $M_{polymer}$ is the weight of input copolymers in NMR test. The ideal PEG layer densities of the PLGA-PEG-NPs and PLA-PEG-NPs were calculated by assuming that all the PEGs are in NP PEG layer as:

$$\frac{1}{A} = \frac{M_{polymer} \times f \times N_A}{MW_{PEG}} \times \frac{\rho \times \frac{4}{3} \pi \left(\frac{d_{DLS}}{2}\right)^3}{M_{polymer} \times 4\pi \left(\frac{d_{DLS}}{2}\right)^2},$$

in which f is the weight fraction of PEG in PLGA-PEG-NPs (20%) and PLA-PEG-NPs (23.8%).

Biodistribution study.

Female BALB/c mice at 7-week old were injected orthotopically with 50 μ L of 1×10^7 4T1 cells/mL in saline into the mammary fat pad on the right flank. Tumor size was measured and calculated by $V = 0.5 \times \text{width}^2 \times \text{length}$. When the tumor size reached 200-350 mm³, the mice were randomly divided into 5 groups and i.v. injected with 150 μ L of DiD-labeled PLGA-PEG-NPs, PLGA-TPEG-NPs-20, PLA-PEG-NPs, or PLA-TPEG-NPs-10 at 10 mg/mL in saline. The fluorescence intensity of NP solutions was equalized. Mice were imaged at 1, 3, 6, 24 and 48 h post-injection using IVIS Lumina XR. After 48 h, mice were sacrificed and their blood, brains, lungs, hearts, livers, spleens, kidneys as well as tumors were collected and imaged with IVIS Lumina XR (605 nm excitation and Cy5.5 emission, 1s exposure). To quantify the NPs in each tissue, all the tissues were weighed and a fraction of the tissues were homogenized. The fluorescence intensities of the homogenized tissue solution were detected *via* a microplate reader.

Statistical analysis.

Data are presented as mean $\pm$ SD. Statistical differences among experimental groups were analyzed using one-way analysis of variance (ANOVA) followed by two-tailed Student's t-test; $P < 0.05$ was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

This work was supported by grants from Drexel Clinical & Translational Research Institute (CTRI); ACPMP Research Foundation to W.B.B. and H.C., Commonwealth Universal Research Enhancement grant from Pennsylvania Department of Health to C.Y. Li., H.C., and W.B.B., and National Institute of Allergy and Infectious Diseases of the National Institutes of Health under Award Number R21AI133372. The authors thank Prof. Irwin Chaiken, Prof. Michael J. Bouchard, Dr. Rachna Niraj, and Nicholas J. Duchemin for providing the ITC facility and trainings to use ITC and isolate liver cells. The authors thank Dr. Hsin-Yao Tang at the Wistar Institute Proteomics and Metabolomics Facility for his assistance on the analysis of proteomics data. The authors also thank Prof. Tonya L. Kuhl for the helpful discussion about grafted PEG on lipid membrane surfaces.

REFERENCES

1. Tsoi KM; MacParland SA; Ma XZ; Spetzler VN; Echeverri J; Ouyang B; Fadel SM; Sykes EA; Goldaracena N; Kathis JM; Conneely JB; Alman BA; Selzner M; Ostrowski MA; Adeyi OA; Zilman A; McGilvray ID; Chan WCW Mechanism of Hard-Nanomaterial Clearance by the Liver. *Nat. Mater* 2016, 15, 1212–1221. [PubMed: 27525571]
2. Chen FF; Wang GK; Griffin JI; Brenneman B; Banda NK; Holers VM; Backos DS; Wu LP; Moghimi SM; Simberg D Complement Proteins Bind to Nanoparticle Protein Corona and Undergo Dynamic Exchange in Vivo. *Nat. Nanotechnol* 2017, 12, 387–393. [PubMed: 27992410]
3. Owens DE; Peppas NA Opsonization, Biodistribution, and Pharmacokinetics of Polymeric Nanoparticles. *Int. J. Pharm* 2006, 307, 93–102. [PubMed: 16303268]
4. Gref R; Minamitake Y; Peracchia MT; Trubetskoy V; Torchilin V; Langer R Biodegradable Long-Circulating Polymeric Nanospheres. *Science* 1994, 263, 1600–1603. [PubMed: 8128245]
5. Zhang L; Cao Z; Bai T; Carr L; Ella-Menye JR; Irvin C; Ratner BD; Jiang S Zwitterionic Hydrogels Implanted in Mice Resist the Foreign-Body Reaction. *Nat. Biotechnol* 2013, 31, 553–556. [PubMed: 23666011]
6. Bertrand N; Grenier P; Mahmoudi M; Lima EM; Appel EA; Dormont F; Lim JM; Karnik R; Langer R; Farokhzad OC Mechanistic Understanding of in Vivo Protein Corona Formation on Polymeric Nanoparticles and Impact on Pharmacokinetics. *Nat. Commun* 2017, 8, 8. [PubMed: 28364116]
7. Suk JS; Xu QG; Kim N; Hanes J; Ensign LM Pegylation as a Strategy for Improving Nanoparticle-Based Drug and Gene Delivery. *Adv. Drug Deliv. Rev.* 2016, 99, 28–51. [PubMed: 26456916]
8. Hamad I; Al-Hanbali O; Hunter AC; Rutt KJ; Andresen TL; Moghimi SM Distinct Polymer Architecture Mediates Switching of Complement Activation Pathways at the Nanosphere-Serum Interface: Implications for Stealth Nanoparticle Engineering. *ACS Nano* 2010, 4, 6629–6638. [PubMed: 21028845]
9. Jackson MA; Werfel TA; Curvino EJ; Yu F; Kavanaugh TE; Sarett SM; Dockery MD; Kilchrist KV; Jackson AN; Giorgio TD; Duvall CL Zwitterionic Nanocarrier Surface Chemistry Improves Sirna Tumor Delivery and Silencing Activity Relative to Polyethylene Glycol. *ACS Nano* 2017, 11, 5680–5696. [PubMed: 28548843]
10. Sofia SJ; Premnath V; Merrill EW Poly(Ethylene Oxide) Grafted to Silicon Surfaces: Grafting Density and Protein Adsorption. *Macromolecules* 1998, 31, 5059–5070. [PubMed: 9680446]
11. Cabral H; Matsumoto Y; Mizuno K; Chen Q; Murakami M; Kimura M; Terada Y; Kano MR; Miyazono K; Uesaka M; Nishiyama N; Kataoka K Accumulation of Sub-100 Nm Polymeric Micelles in Poorly Permeable Tumours Depends on Size. *Nat. Nanotechnol* 2011, 6, 815–823. [PubMed: 22020122]
12. Kwon GS; Kataoka K Block Copolymer Micelles as Long-Circulating Drug Vehicles. *Adv. Drug Deliv. Rev.* 1995, 16, 295–309.
13. Fang C; Shi B; Pei YY; Hong MH; Wu J; Chen HZ In Vivo Tumor Targeting of Tumor Necrosis Factor-Alpha-Loaded Stealth Nanoparticles: Effect of Mepeg Molecular Weight and Particle Size. *Eur. J. Pharm. Sci* 2006, 27, 27–36. [PubMed: 16150582]
14. Tockary TA; Osada K; Chen Q; Machitani K; Dirisala A; Uchida S; Nomoto T; Toh K; Matsumoto Y; Itaka K; Nitta K; Nagayama K; Kataoka K Tethered Peg Crowdedness Determining Shape and Blood Circulation Profile of Polyplex Micelle Gene Carriers. *Macromolecules* 2013, 46, 6585–6592.

15. Walkey CD; Olsen JB; Guo HB; Emili A; Chan WCW Nanoparticle Size and Surface Chemistry Determine Serum Protein Adsorption and Macrophage Uptake. *J. Am. Chem. Soc* 2012, 134, 2139–2147. [PubMed: 22191645]
16. Gref R; Luck M; Quellec P; Marchand M; Dellacherie E; Harnisch S; Blunk T; Muller RH ‘Stealth’ Corona-Core Nanoparticles Surface Modified by Polyethylene Glycol (Peg): Influences of the Corona (Peg Chain Length and Surface Density) and of the Core Composition on Phagocytic Uptake and Plasma Protein Adsorption. *Colloid Surf. B-Biointerfaces* 2000, 18, 301–313.
17. Uchida K; Otsuka H; Kaneko M; Kataoka K; Nagasaki Y A Reactive Poly(Ethylene Glycol) Layer to Achieve Specific Surface Plasmon Resonance Sensing with a High S/N Ratio: The Substantial Role of a Short Underbrushed Peg Layer in Minimizing Nonspecific Adsorption. *Anal. Chem* 2005, 77, 1075–1080. [PubMed: 15858988]
18. Matsumoto M; Matsusaki M; Akashi M Preparation of Biodegradable Peptide Nanospheres with Hetero Peg Brush Surfaces. *Macromol. Biosci* 2014, 14, 142–150. [PubMed: 24106226]
19. Nissinen T; Nakki S; Laakso H; Kuciauskas D; Kaupinis A; Kettunen MI; Liimatainen T; Hyvonen M; Valius M; Grohn O; Lehto VP Tailored Dual Pegylation of Inorganic Porous Nanocarriers for Extremely Long Blood Circulation in Vivo. *ACS Appl. Mater. Interfaces* 2016, 8, 32723–32731. [PubMed: 27934159]
20. Unsworth LD; Sheardown H; Brash JL Protein Resistance of Surfaces Prepared by Sorption of End-Thiolated Poly(Ethylene Glycol) to Gold: Effect of Surface Chain Density. *Langmuir* 2005, 21, 1036–1041. [PubMed: 15667186]
21. Liu G; Chen Y; Zhang G; Yang S Protein Resistance of (Ethylene Oxide)_N Monolayers at the Air/Water Interface: Effects of Packing Density and Chain Length. *Phys. Chem. Chem. Phys* 2007, 9, 6073–6082. [PubMed: 18167582]
22. Vanderah DJ; La HL; Naff J; Silin V; Rubinson KA Control of Protein Adsorption: Molecular Level Structural and Spatial Variables. *J. Am. Chem. Soc* 2004, 126, 13639–13641. [PubMed: 15493920]
23. Wu C How Does a Polymer Brush Repel Proteins? *Chin. J. Polym. Sci* 2014, 32, 1575–1580.
24. de Gennes PG Conformations of Polymers Attached to an Interface. *Macromolecules* 1980, 13, 1069–1075.
25. Jeon SI; Andrade JD Protein—Surface Interactions in the Presence of Polyethylene Oxide: Ii. Effect of Protein Size. *J. Colloid Interface Sci.* 1991, 142, 159–166.
26. Fang F; Szleifer I Kinetics and Thermodynamics of Protein Adsorption: A Generalized Molecular Theoretical Approach. *Biophys. J* 2001, 80, 2568–2589. [PubMed: 11371435]
27. Geng Y; Dalhaimer P; Cai SS; Tsai R; Tewari M; Minko T; Discher DE Shape Effects of Filaments Versus Spherical Particles in Flow and Drug Delivery. *Nat. Nanotechnol* 2007, 2, 249–255. [PubMed: 18654271]
28. Merkel TJ; Chen K; Jones SW; Pandya AA; Tian SM; Napier ME; Zamboni WE; DeSimone JM The Effect of Particle Size on the Biodistribution of Low-Modulus Hydrogel Print Particles. *J. Control. Release* 2012, 162, 37–44. [PubMed: 22705460]
29. Anselmo AC; Zhang MW; Kumar S; Vogus DR; Menegatti S; Helgeson ME; Mitragotri S Elasticity of Nanoparticles Influences Their Blood Circulation, Phagocytosis, Endocytosis, and Targeting. *ACS Nano* 2015, 9, 3169–3177. [PubMed: 25715979]
30. Qi H; Zhou H; Tang Q; Lee JY; Fan Z; Kim S; Staub MC; Zhou T; Mei S; Han L; Pochan DJ; Cheng H; Hu W; Li CY Block Copolymer Crystalsomes with an Ultrathin Shell to Extend Blood Circulation Time. *Nat. Commun.* 2018, 9, 3005. [PubMed: 30068976]
31. Sun X; Wang G; Zhang H; Hu S; Liu X; Tang J; Shen Y The Blood Clearance Kinetics and Pathway of Polymeric Micelles in Cancer Drug Delivery. *ACS Nano* 2018, 12, 6179–6192.
32. Lu Y; Yue ZG; Xie JB; Wang W; Zhu H; Zhang ES; Cao ZQ Micelles with Ultralow Critical Micelle Concentration as Carriers for Drug Delivery. *Nat. Biomed. Eng* 2018, 2, 318–325.
33. Hu CM; Zhang L; Aryal S; Cheung C; Fang RH; Zhang L Erythrocyte Membrane-Camouflaged Polymeric Nanoparticles as a Biomimetic Delivery Platform. *Proc. Natl. Acad. Sci. U. S. A* 2011, 108, 10980–10985. [PubMed: 21690347]

34. Rodriguez PL; Harada T; Christian DA; Pantano DA; Tsai RK; Discher DE Minimal “Self” Peptides That Inhibit Phagocytic Clearance and Enhance Delivery of Nanoparticles. *Science* 2013, 339, 971–975. [PubMed: 23430657]
35. Schottler S; Becker G; Winzen S; Steinbach T; Mohr K; Landfester K; Mailander V; Wurm FR Protein Adsorption Is Required for Stealth Effect of Poly(Ethylene Glycol)- and Poly(Phosphoester)-Coated Nanocarriers. *Nat. Nanotechnol* 2016, 11, 372–377. [PubMed: 26878141]
36. Cheng J; Teply BA; Sherifi I; Sung J; Luther G; Gu FX; Levy-Nissenbaum E; Radovic-Moreno AF; Langer R; Farokhzad OC Formulation of Functionalized Plga-Peg Nanoparticles for in Vivo Targeted Drug Delivery. *Biomaterials* 2007, 28, 869–876. [PubMed: 17055572]
37. Zhang Y; Huo M; Zhou X; Xie S Pksolver: An Add-in Program for Pharmacokinetic and Pharmacodynamic Data Analysis in Microsoft Excel. *Comput. Methods Programs Biomed.* 2010, 99, 306–314. [PubMed: 20176408]
38. Zhou H; Fan ZY; Deng JJ; Lemons PK; Arhontoulis DC; Bowne WB; Cheng H Hyaluronidase Embedded in Nanocarrier Peg Shell for Enhanced Tumor Penetration and Highly Efficient Antitumor Efficacy. *Nano Letters* 2016, 16, 3268–3277. [PubMed: 27057591]
39. Yang B; Lowe JP; Schweins R; Edler KJ Small Angle Neutron Scattering Studies on the Internal Structure of Poly(Lactide-Co-Glycolide)-Block-Poly(Ethylene Glycol) Nanoparticles as Drug Delivery Vehicles. *Biomacromolecules* 2015, 16, 457–464. [PubMed: 25539145]
40. Perry JL; Reuter KG; Kai MP; Herlihy KP; Jones SW; Luft JC; Napier M; Bear JE; DeSimone JM Pegylated Print Nanoparticles: The Impact of Peg Density on Protein Binding, Macrophage Association, Biodistribution, and Pharmacokinetics. *Nano Letters* 2012, 12, 5304–5310. [PubMed: 22920324]
41. Szleifer I Statistical Thermodynamics of Polymers near Surfaces. *Curr. Opin. Colloid Interface Sci.* 1996, 1, 416–423.
42. Kuhl TL; Leckband DE; Lasic DD; Israelachvili JN Modulation of Interaction Forces between Bilayers Exposing Short-Chained Ethylene Oxide Headgroups. *Biophys. J* 1994, 66, 1479–1488. [PubMed: 8061197]
43. Jeppesen C; Wong JY; Kuhl TL; Israelachvili JN; Mullah N; Zalipsky S; Marques CM Impact of Polymer Tether Length on Multiple Ligand-Receptor Bond Formation. *Science* 2001, 293, 465–468. [PubMed: 11463908]
44. Xu QG; Ensign LM; Boylan NJ; Schon A; Gong XQ; Yang JC; Lamb NW; Cai ST; Yu T; Freire E; Hanes J Impact of Surface Polyethylene Glycol (Peg) Density on Biodegradable Nanoparticle Transport in Mucus Ex Vivo and Distribution in Vivo. *ACS Nano* 2015, 9, 9217–9227. [PubMed: 26301576]
45. Brandl F; Bertrand N; Lima EM; Langer R Nanoparticles with Photoinduced Precipitation for the Extraction of Pollutants from Water and Soil. *Nat. Commun* 2015, 6, 10.
46. Tenzer S; Docter D; Kuharev J; Musyanovych A; Fetz V; Hecht R; Schlenk F; Fischer D; Kiouptsi K; Reinhardt C; Landfester K; Schild H; Maskos M; Knauer SK; Stauber RH Rapid Formation of Plasma Protein Corona Critically Affects Nanoparticle Pathophysiology. *Nat. Nanotechnol* 2013, 8, 772–U1000. [PubMed: 24056901]
47. Lundqvist M; Stigler J; Elia G; Lynch I; Cedervall T; Dawson KA Nanoparticle Size and Surface Properties Determine the Protein Corona with Possible Implications for Biological Impacts. *Proc. Natl. Acad. Sci. U. S. A* 2008, 105, 14265–14270. [PubMed: 18809927]
48. Freire E; Mayorga OL; Straume M Isothermal Titration Calorimetry. *Anal. Chem* 1990, 62, 950A–959A.
49. McPherson T; Kidane A; Szleifer I; Park K Prevention of Protein Adsorption by Tethered Poly(Ethylene Oxide) Layers: Experiments and Single-Chain Mean-Field Analysis. *Langmuir* 1998, 14, 176–186.
50. Park JK; Utsumi T; Seo YE; Deng Y; Satoh A; Saltzman WM; Iwakiri Y Cellular Distribution of Injected Plga-Nanoparticles in the Liver. *Nanomed.-Nanotechnol. Biol. Med* 2016, 12, 1365–1374.
51. Maslak E; Gregorius A; Chlopicki S Liver Sinusoidal Endothelial Cells (Lsecs) Function and Nafld; No-Based Therapy Targeted to the Liver. *Pharmacol. Rep* 2015, 67, 689–694. [PubMed: 26321269]

52. Kamps J; Morselt HWM; Swart PJ; Meijer DKF; Scherphof GL Massive Targeting of Liposomes, Surface-Modified with Anionized Albumins, to Hepatic Endothelial Cells. *Proc. Natl. Acad. Sci. U. S. A* 1997, 94, 11681–11685. [PubMed: 9326670]
53. Carambia A; Freund B; Schwinge D; Bruns OT; Salmen SC; Ittrich H; Reimer R; Heine M; Huber S; Waurisch C; Eychmuller A; Wraith DC; Korn T; Nielsen P; Weller H; Schramm C; Luth S; Lohse AW; Heeren J; Herkel J Nanoparticle-Based Autoantigen Delivery to Treg-Inducing Liver Sinusoidal Endothelial Cells Enables Control of Autoimmunity in Mice. *J. Hepatol* 2015, 62, 1349–1356. [PubMed: 25617499]
54. Campbell F; Bos FL; Sieber S; Arias-Alpizar G; Koch BE; Huwyler J; Kros A Directing Nanoparticle Biodistribution through Evasion and Exploitation of Stab2-Dependent Nanoparticle Uptake. *ACS Nano* 2018.
55. Saha K; Rahimi M; Yazdani M; Kim ST; Moyano DF; Hou S; Das R; Mout R; Rezaee F; Mahmoudi M; Rotello VM Regulation of Macrophage Recognition through the Interplay of Nanoparticle Surface Functionality and Protein Corona. *ACS Nano* 2016, 10, 4421–4430. [PubMed: 27040442]
56. Dos Santos N; Allen C; Doppen AM; Anantha M; Cox KAK; Gallagher RC; Karlsson G; Edwards K; Kenner G; Samuels L; Webb MS; Bally MB Influence of Poly(Ethylene Glycol) Grafting Density and Polymer Length on Liposomes: Relating Plasma Circulation Lifetimes to Protein Binding. *Biochim. Biophys. Acta-Biomembr* 2007, 1768, 1367–1377.
57. Torchilin VP; Omelyanenko VG; Papisov MI; Bogdanov AA; Trubetskoy VS; Herron JN; Gentry CA Poly(Ethylene Glycol) on the Liposome Surface: On the Mechanism of Polymer-Coated Liposome Longevity. *Biochim. Biophys. Acta-Biomembr* 1994, 1195, 11–20.
58. Zhou H; Fan ZY; Lemons PK; Cheng H A Facile Approach to Functionalize Cell Membrane-Coated Nanoparticles. *Theranostics* 2016, 6, 1012–1022. [PubMed: 27217834]
59. Hu CMJ; Fang RH; Wang KC; Luk BT; Thamphiwatana S; Dehaini D; Nguyen P; Angsantikul P; Wen CH; Kroll AV; Carpenter C; Ramesh M; Qu V; Patel SH; Zhu J; Shi W; Hofman FM; Chen TC; Gao WW; Zhang K, et al. Nanoparticle Biointerfacing by Platelet Membrane Cloaking. *Nature* 2015, 526, 118–121. [PubMed: 26374997]
60. Peer D; Karp JM; Hong S; Farokhzad OC; Margalit R; Langer R Nanocarriers as an Emerging Platform for Cancer Therapy. *Nat. Nanotechnol* 2007, 2, 751–760. [PubMed: 18654426]
61. Desai N; Trieu V; Yao ZW; Louie L; Ci S; Yang A; Tao CL; De T; Beals B; Dykes D; Noker P; Yao R; Labao E; Hawkins M; Soon-Shiong P Increased Antitumor Activity, Intratumor Paclitaxel Concentrations, and Endothelial Cell Transport of Cremophor-Free, Albumin-Bound Paclitaxel, Abi-007, Compared with Cremophor-Based Paclitaxel. *Clin. Cancer Res.* 2006, 12, 1317–1324. [PubMed: 16489089]
62. Wilhelm S; Tavares AJ; Dai Q; Ohta S; Audet J; Dvorak HF; Chan WCW Analysis of Nanoparticle Delivery to Tumours. *Nat. Rev. Mater* 2016, 1, 12.
63. Gstraunthaler G Alternatives to the Use of Fetal Bovine Serum: Serum-Free Cell Culture. *Altex* 2003, 20, 275–281. [PubMed: 14671707]
64. Heald CR; Stolnik S; Kujawinski KS; De Matteis C; Garnett MC; Illum L; Davis SS; Purkiss SC; Barlow RJ; Gellert PR Poly(Lactic Acid)-Poly(Ethylene Oxide) (Pla-Peg) Nanoparticles: Nmr Studies of the Central Solidlike Pla Core and the Liquid Peg Corona. *Langmuir* 2002, 18, 3669–3675.
65. Garcia-Fuentes M; Torres D; Martin-Pastor M; Alonso MJ Application of Nmr Spectroscopy to the Characterization of Peg-Stabilized Lipid Nanoparticles. *Langmuir* 2004, 20, 8839–8845. [PubMed: 15379515]

Figure 1. Long blood circulation half-lives of PLGA-PEG-NPs with a dynamic topographical structure of PEG shell (PLGA-TPEG-NPs).

(a) Schematic illustration of PLGA-TPEG-NP preparation. PLGA-TPEG-NP-20 indicates that the NPs were prepared with 20% of PLGA-PEG-MAL and 80% of PLGA-PEG, and further modified with methoxy PEG_{2K}-thiol to generate a hierarchical shell structure. (b) A representative transmission electron microscopy (TEM) image of PLGA-TPEG-NPs-20 (Scale bar: 100 nm). (c) Blood circulation of PLGA-TPEG-NPs measured by fluorescence intensity of NPs in the plasma. The NPs were prepared with 0%, 10%, 20%, 40%, 60%, 80%, and 100% PLGA-PEG-MAL. Values indicate mean $\pm$ SD ($n = 6$, from two independent experiments). All NPs were fluorescently labeled by encapsulating 1,1-

dioctadecyl-3,3,3-tetramethylindocarbocyanine perchlorate (DiD). (d) Circulation half-lives of PLGA-TPEG-NPs obtained by fitting the circulation profile data (c) into a one-compartment model *via* PKSolver. Values indicate mean $\pm$ SD (n = 6). (e) Schematic illustration of the proposed three-dimensional PEG structure on NP surfaces with an increased PEG density in the outer PEG layer. The primary layer of PEG forms a dense polymer brush which repels proteins sterically. The outer layer of PEG changes from mushroom to brush regime as PLGA-PEG-MAL percentage increases. Within each blob, the PEG chain behaves as a free polymer in a good solvent. (f) Calculated average distance of neighboring chains (D) and thickness (L) of the outer PEG layer, which determines the topographical structure of PEG shells. The calculation followed de Gennes' model of grafted polymers on a flat surface in a good solvent. To simplify the estimation, $L \approx Na^{5/3} D^{-2/3}$ was applied when $D < R_F$. Values indicate mean $\pm$ SD (n = 3). Two methods were used to calculate D and L : (1) a standard method using hydrated NP size from DLS to calculate NP concentration and surface area; and (2) a method using dehydrated NP size from TEM to calculate NP concentration.

Figure 2. Isothermal titration calorimetry (ITC) study of protein binding on NPs.

The dynamic topographical structure of PEG shell reduces the affinity of protein binding on NPs. (a-d) Representative ITC data. Graphs show integrated heat of each titration (■) with a corresponding fitted curve based on the one-site binding model for (a) PLGA-NPs, (b) PLGA-PEG-NPs, (c) PLGA-TPEG-NP-20, and (d) PLGA-TPEG-NP-100. (e,f), Calculated stoichiometry (Na) and binding affinity (Ka) for the indicated samples. Values indicate mean $\pm$ SD ($n = 3$). ***, $P < 0.001$.

Figure 3. Dynamic topographical structure of PEG shells significantly reduced the uptake of PEGylated NPs by endothelial cells in the liver.

Liver cells are stained with CD146 and CD68. (a) Representative histogram of NP internalization of all cells in the livers collected from mice 4 h after tail vein injection of saline, PLGA-PEG-NP or PLGA-TPEG-NP-20. (b) Percentage of cells that internalized NPs (DiD⁺) within hepatocytes, endothelial cells, Kupffer cells and other cells. (c-f) Representative histograms of NP internalization of endothelial cells, Kupffer cells, hepatocytes, and other cells in the livers. (g) Relative mean fluorescence intensity (MFI) of different liver cells. (h) Relative MFI of hepatocytes that internalized NPs or not (NP⁺/NP⁻).

(i) Relative MFI of other cells that internalized NPs or not (NP^+/NP^-). Values indicate mean $\pm$ SD (n = 5 from three independent experiments). *, $P < 0.05$, ***, $P < 0.001$.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 4. Long blood circulation of PLA-PEG-NPs with a controlled topographical structure. (a) Blood circulation of DiD-labeled PLA-TPEG-NPs prepared with 0%, 5%, 10% and 20% PLA-PEG-MAL. (b) Circulation half-lives of PLA-TPEG-NPs obtained by fitting the circulation profile data (a) into a one-compartment model *via* PKSolver. Values indicate mean $\pm$ SD ($n = 4-6$, from two independent experiments). **, $P < 0.01$.

Figure 5. Highly efficient NP accumulation in tumors from long circulating NPs. 4T1 tumor-bearing mice were administered with either (1) saline, (2) PLGA-PEG-NPs, (3) PLGA-TPEG-NPs-20, (4) PLA-PEG-NPs or (5) PLA-TPEG-NPs-10 *via* tail vein injection. All NPs were labeled with DiD. (a) IVIS fluorescence images of mice at 3, 6, 24 and 48 h post-injection of NPs or saline control. (b) IVIS fluorescence image of tissues 48 h post-injection. (c) Quantification of NP biodistribution in mice by measuring the fluorescence intensity of homogenized tissues that were collected at 48 h post-injection of NPs. Values indicate mean $\pm$ SD ($n = 3$). ***, $P < 0.001$, **, $P < 0.01$.