Published in final edited form as:

Nano Lett. 2018 October 10; 18(10): 6164–6174. doi:10.1021/acs.nanolett.8b01892.

Leutosome: A biomimetic nanoplatform integrating plasma membrane components of leukocytes and tumor cells for remarkably enhanced solid tumor homing

Hongliang He^{1,2}, Chunqing Guo^{3,4,5}, Jing Wang², William J. Korzun⁶, Xiang-Yang Wang^{3,4,5}, Shobha Ghosh^{2,*}, and Hu Yang^{1,5,7,*}

¹Department of Chemical and Life Science Engineering, Virginia Commonwealth University, Richmond, Virginia 23219, United States

²Department of Internal Medicine, Virginia Commonwealth University, Richmond, Virginia 23298, United States

³Department of Human Molecular Genetics, Virginia Commonwealth University, Richmond, Virginia 23298, United States

⁴Institute of Molecular Medicine, Virginia Commonwealth University, Richmond, Virginia 23298, United States

⁵Massey Cancer Center, Virginia Commonwealth University, Richmond, Virginia 23298, United States

⁶Department of Clinical Laboratory Sciences, Virginia Commonwealth University, Richmond, Virginia 23298, United States

⁷Department of Pharmaceutics, Virginia Commonwealth University, Richmond, Virginia 23298, United States

Abstract

Cell membrane-camouflaged nanoparticles have appeared as a promising platform to develop active tumor targeting nanomedicines. To evade the immune surveillance, we designed a composite cell membrane-camouflaged biomimetic nanoplatform, namely leutosome, which is made of liposomal nanoparticles incorporating plasma membrane components derived from both leukocytes (murine J774A.1 cells) and tumor cells (head and neck tumor cells HN12). Exogenous phospholipids were used as building blocks to fuse with two cell membranes to form liposomal nanoparticles. Liposomal nanoparticles made of exogenous phospholipids only or in combination with one type of cell membrane were fabricated and compared. The anticancer drug paclitaxel (PTX) was used to make drug-encapsulating liposomal nanoparticles. Leutosome resembling characteristic plasma membrane components of the two cell membranes were examined and confirmed *in vitro*. A xenograft mouse model of head and neck cancer was used to profile the

*Corresponding Author: shobha@vcu.edu, hyang2@vcu.edu.

Supporting Information

Experimental section, supporting figures.

The authors declare no competing interest.

blood clearance kinetics, biodistribution and antitumor efficacy of the different liposomal nanoparticles. The results demonstrated that leutosome obtained prolonged blood circulation and was most efficient accumulating at the tumor site ($79.1 \pm 6.6\%$ ID per gram of tumor). Similarly, leutosome composed of membrane fractions of B16 melanoma cells and leukocytes (J774A.1) showed prominent accumulation within the B16 tumor, suggesting the generalization of the approach. Furthermore, leutosome was found to most potently inhibit tumor growth while not causing systemic adverse effects.

TOC Graphic

Keywords

active targeting; nanoparticles; tumor microenvironment; leukocytes; tumor cells; cell membrane camouflage

Although tremendous efforts have been committed to developing tumor-targeted drug delivery systems, therapeutic outcomes are plagued with their inadequate circulation and low targeting specificity.^{1–4} Recently cell membrane camouflaged-nanoparticles have emerged as a biomimetic platform for drug delivery.^{5–7} Cell membrane of interest can be extracted and coated onto nanoparticles or used as building blocks to form nanoparticles on its own or with other polymeric entities. Cell membranes of erythrocytes,^{8–10} platelets,^{11, 12} leukocytes^{13–17} and tumor cells^{18–25} have been exploited to endow nanoparticles with surface characteristics of the source cells. In addition, cell membrane camouflage does not require the use of labor-intensive complicated bioconjugation methods, making itself an appealing approach for the generation of biofunctionalized nanoparticles.^{6, 26–28}

Leukocytes, the critical cells of the immune system²⁹, significantly prolong blood circulation of the formulations when they are exercised as cellular vehicles or provide their plasma membrane to disguise nanoparticles.^{30–39} However, desirable therapeutic efficacy has not been fully attained by leukocyte-based drug delivery systems, due in part to incompetent tumor cell targeting.^{40–42} Therefore, rational optimization of leukocyte-based drug delivery systems is to enhance their affinity towards tumor cells and subsequent uptake by tumor cells. Use of tumor cell membrane camouflage to develop next-generation anticancer diagnostic and therapeutic agents is substantiated by a large body of evidence to show that tumor cell membrane-camouflaged nanoparticles preferentially home to the tumor via the so-called homotypic tumor targeting.^{18–25, 43, 44} The tumor cell membrane-camouflaged nanoparticles reported so far, however, do not seem stealth enough to evade the immune surveillance as evidenced by their rapid phagocytic clearance from the blood and significant accumulation in the mononuclear phagocytic system (MPS). This limitation is

attributed to the presence of tumor-specific antigens on the surface. Despite the fact that the immunostimulatory property associated with tumor cell membranes has been harnessed to boost immune response for cancer immunotherapy,^{20, 45, 46} such property becomes least desirable in regards to the delivery of chemotherapeutics to solid tumors.

When it comes to the development of cell membrane-camouflaged nanoparticles for cancer theranostics, it is crucial for cell membrane-camouflaged nanoparticles to possess not only the ability to thwart detection by the immune system for extended blood circulation but also the homotypic tumor targeting. The combination of membrane proteins of different cell types is thought to bestow those multiple biofunctions inherited from those parental cells to achieve a chimeric membrane camouflage.^{36, 47} For instance, composite erythrocyte and platelet cell membrane camouflaged-nanoparticles exhibit erythrocyte-mediated long circulation and platelet-specific targeting property.⁴⁸ Herein, we hypothesized that incorporation of leukocyte membrane components would aid tumor cell membrane-camouflaged nanoparticles to obtain prolonged blood circulation and minimize the MPS uptake, thus leading to enhanced solid tumor homing. In this work, we designed a composite cell membrane-camouflaged biomimetic nanopatform, namely leutosome, which is made of liposomal nanoparticles incorporating plasma membrane components derived from both leukocytes and tumor cells (Scheme 1). Murine J774A.1 cells and HN12 tumor cells were selected as source leukocytes and tumor cells, respectively. Exogenous phospholipids were used as building blocks to fuse with two cell membranes to form liposomal nanoparticles given the excellent cell membrane fusion property and high loading capacity for poorly water-soluble anticancer drugs. Liposomal nanoparticles made of exogenous phospholipids only or in combination with one type of cell membrane were fabricated and compared with leutosome for tumor targeting specificity *in vitro* and *in vivo*. *In vivo* antitumor efficacy and safety of the paclitaxel (PTX) loaded liposomal nanoparticles was examined as well.

Leukocyte membrane components were incorporated into the tumor cell membrane-camouflaged nanoparticles and tested whether it would aid tumor cell membrane-camouflaged nanoparticles to obtain prolonged blood circulation and minimize the MPS uptake for enhanced solid tumor homing. Leukocyte membrane (L-membrane) and tumor cell membrane (T-membrane) were extracted and purified following the procedures illustrated in Figure S1. To monitor the presence of both cell membrane components, L-membrane and T-membrane were labeled with fluorescent dyes DiO (green) and DiD (red), respectively. DiO-labeled L-membrane and DiD-labeled T-membrane did not spontaneously fuse into nanoscale vesicles until they underwent a series of extrusion after sonication (Figure 1A). The fluorescence images show that before extrusion, the two cell membranes largely stay separate (see separate Red & Green channels). In addition, the TEM images show that they aggregate and exhibit irregular shapes (microscale). They fused and transformed into nanoscale vesicles after extrusion, suggesting the need of extrusion to fuse two membrane fragments into nanoscale vehicles. The internalization of the vesicles by source tumor cells was confirmed with confocal microscopy. The whole cell fluorescence intensities in the green and red channels are equally strong (Figure 1B). The merge channel shows the colocalization (yellow) of DiO-labeled L-membrane and DiD-labeled T-membrane, suggesting that the incubation of cells with composite nanoscale vesicles presented both cell membrane components. The obvious uptake of the composite vesicles by

tumor cells laid the foundation for exploring composite cell membrane-camouflaged liposomes for anticancer drug delivery.

The analysis of cell membrane biomarkers reveals that the composite cell membranes (in the form of either mixture or vesicles) were mostly free of intracellular components such as lamin B, PDI, and cytochrome C found in the nucleus, endoplasmic reticulum and mitochondria, respectively (Figure 2A). They were significantly enriched with non-specific plasma membrane markers such as Na^+/K^+ ATPase α -1, and protein markers specific to source leukocytes such as CD45, CD47, SR-A and mannose receptor, and those specific to source tumor cells such as E-cadherin and folate receptor. Furthermore, composite cell membrane-camouflaged liposomes fully recapitulate the cell membrane biomarkers of leukocytes and tumor cells, suggesting the extracted cell membranes were well-integrated with exogenous phospholipids in leutosome.

The size, surface charge, and morphology of PTX-encapsulating liposomes made of exogenous phospholipids only or in combination with one or two types of cell membranes were characterized. All the liposomal nanoparticles had a similar size range and retained a negatively charged surface. They had various zeta potentials as a result of variation of lipid compositions. Compared with the control liposome containing PTX (PTXL) with a mean size of 162.0 ± 12.4 nm and a polydispersity index of 0.177 ± 0.024 , LM-, TM- and LTM-PTXL do not show obvious deviation in particle size and size distribution (Figures 2B and Figure S2). This result indicates that the extracted cell membranes can act as lipid blocks to integrate with external phospholipids to form stable liposomes. The integration mechanism is different from cell membrane-coated polymeric nanoparticles where cell membrane acts as a coating shield to cover the surface of the polymeric core, leading to particle size increase and the formation of core-shell structure. Paclitaxel (PTX), a poorly water soluble chemotherapeutic agent, was encapsulated into the phospholipids bilayer of liposome. When the drug-to-lipid mass ratio was kept at 1:20 (w/w), both encapsulation efficiency (%) and loading content (mole %) were highest for all liposomal nanoparticles (Figure 2C and 2D).

Compared with the zeta potential of PTXL (-8.1 ± 1.4 mV), the surface charge of the three camouflaged liposomes decreased remarkably. The surface charge reduction was attributed to the shielding effect of negatively charged cell membrane-associated proteins on the surface of camouflaged liposomes.^{14, 15, 21} In addition, leutosome LTM-PTXL was found to have an intermediate zeta potential (-21.6 ± 3.9 mV), which is more negative than that of TM-PTXL (-14.4 ± 2.2 mV) but less negative than that of LM-PTXL (-30.1 ± 0.8 mV). This is viewed as evidence of successful integration/fusion of two cellular membranes into leutosome. As seen in the TEM images, all the liposomal nanoparticles display typical liposomal characteristics with particle sizes in agreement with the DLS data. The liposomal formulations retained colloidal stability for at least one week after storage at 4°C. They did not show significant changes in size and zeta potential (Figure 2E and 2F). The *in vitro* PTX release results (Figure 2G) show that all the liposomal nanoparticles released PTX at a similar rate, which, however, was much slower than Taxol. For instance, 80% of the drug was released from Taxol in 8 h while only 45% of the drug was released from liposomal nanoparticles within the same time frame.

A co-culture cell model consisting of leukocytes (murine J774A.1 cells) and tumor cells (YFP-expressing HN12 cells) was used to examine the effect of different cell plasma membranes (leukocytes or tumor cell) on cellular uptake of liposomal nanoparticles at nontoxic concentrations (Figure S3). YFP-expressing HN12 cells express strong green fluorescence and can be distinctly differentiated from murine J774A.1 cells that only show nuclear DAPI staining. DiI-labeled liposomes (red) fluorescence signal in each cell population was qualitatively characterized with confocal imaging (Figure 3A) and quantified with flow cytometry regarding mean fluorescence intensity (MFI) of DiI per cell type (Figure 3B).

DiIL was made of synthetic phospholipids only. Leukocytes J774A.1 showed 2.9 times higher phagocytic property for DiIL than the tumor cells ($p < 0.01$), suggesting the highly phagocytic property for leukocytes. Compared with DiIL, LM-DiIL had similar uptake in HN12 cells, but a 2.8-fold reduction was noted in J774A.1 cells. The decreased uptake by J774A.1 cells was attributed to the incorporation of the host leukocyte membrane into LM-DiIL, which helped the nanoparticles mimic host leukocytes to reduce phagocytosis ($p < 0.01$). The inclusion of tumor cell membrane significantly increased cellular uptake of TM-DiIL in HN12 cells, a 4.3-fold increase compared to DiIL. Such a dramatic boost in cellular uptake is attributed to the homotypic binding ability endowed with the tumor cell membrane. The similar observation was reported by other groups.^{19, 23, 24} Nonetheless, tumor cell membrane also enhanced uptake of TM-DiIL in J774A.1 cells by 1.6-fold ($p < 0.01$), possibly provoked by tumor antigens present on TM-DiIL. In contrast, after the incorporation of both leukocyte membrane and tumor cell membrane into the DiIL, the resulting DiI-loaded leutosome (LTM-DiIL) not only showed the 3.9 times ($p < 0.01$) higher uptake in homotypic tumor cell, but showed the 2.4 times ($p < 0.01$) lower uptake in leukocytes that was similar to the uptake of LT-DiIL in leukocytes. The data above suggest that leutosome (LTM-DiIL) gained tumor specificity and reduced engulfment by leukocytes. Besides, all the tested liposomal nanoparticles did not show any apparent cytotoxicity (Figure S3).

Regardless of phospholipids concentration and cell membrane type, all empty liposomal nanoparticles (without PTX loading) exhibit good cytocompatibility when phospholipids concentration is below 2600 μM (Figure 4A). The dose-dependent antitumor effect of the four PTX-loaded liposomal nanoparticles was evaluated over a broad range of PTX equivalent concentrations up to 230 μM (Figure 4B), at which the phospholipids concentration was nontoxic. Compared to IC₅₀ for free Taxol ($15.5 \pm 1.0 \mu\text{M}$), PTXL had an IC₅₀ of $21.8 \pm 0.9 \mu\text{M}$ (1.4-fold less potent than Taxol), and the IC₅₀ of LM-PTXL was $35.5 \pm 1.7 \mu\text{M}$ (2.3-fold less potent than Taxol). Given that conventional liposomes and Taxol do not deviate significantly in cellular uptake kinetics,^{49, 50} we attribute the higher IC₅₀s of PTXL and LM-PTXL to the slower release of PTX. PTX in Taxol can be released at a much faster rate and diffused freely into HN12 cells, exerting an immediate inhibitory effect. In contrast, PTX was slowly released from PTXL and LM-PTXL upon internalization, causing a delayed inhibitory effect and accounting for the IC₅₀ increase. Among the tested liposomal nanoparticles, TM-PTXL and LTM-PTXL showed the most potent inhibitory effect as evidenced by significantly reduced IC₅₀s ($2.6 \pm 0.4 \mu\text{M}$ for LM-PTXL and LTM-PTXL for $3.3 \pm 0.3 \mu\text{M}$). Their enhanced antitumor activity was

presumably due to the dramatically elevated cellular uptake as opposed to the other formulations as observed. Nonetheless, *in vitro* PTX release kinetics between TM-PTXL and LTM-PTXL was similar to one another, making them exhibit similar IC50s *in vitro*.

The biocompatibility of the tested empty liposomal nanoparticles was first confirmed *in vivo*. A high dose of liposomal nanoparticles (320 mg phospholipids/kg) did not cause adverse systemic toxicity effects based on examination of pro-inflammatory cytokines IL-1 β and TNF- α in serum (Figure S4) as well as histological examination of major organs (Figure S5).

After the intravenous administration of DiI-labeled liposomal nanoparticles, the accumulation in both monocytes and neutrophils, abundant phagocytes in the blood of mice, were quantified. Significant uptake of TM-DiIL and DiIL by monocytes and neutrophils was observed (Figure 5, Figures S6-S9). Nonetheless, uptake of LM-DiIL and LTM-DiIL by monocytes and neutrophils was dramatically reduced, suggesting that the camouflage of leukocyte membrane helped reduce the clearance of nanoparticles by the phagocytes in the MPS.

A xenograft mouse model of head and neck cancer was established by implanting YFP-expressing HN12 cells to the flank of athymic nude mice and used to examine the biodistribution and plasma clearance kinetics of the liposomal nanoparticles aided with near-infrared imaging. DiR-labeled liposomal nanoparticles (DiRL, LM-DiRL, TM-DiRL, and LTM-DiRL) were used in this study. Whole-body live imaging was conducted to track near IR signal in the mice over the course of 48-h after they received one dose of liposomal vehicles (Figure 6A). Due to the lack of active tumor targeting feature, DiRL showed limited accumulation in the tumor, and it primarily accumulated in the liver, spleen, and lung, which is consistent with the typical biodistribution characteristics of liposomes-based drug vehicles. It relied on enhanced permeability and retention (EPR) effect to gain passive accumulation in the tumor.^{51, 52} Although the fluorescence signal intensity increased over time in general, only marginally enhancement in tumor accumulation of LM-DiRL was seen. In contrast, both TM-DiRL and LTM-DiRL showed strong accumulation in the tumor as early as at 6 h post-injection, and stronger signals were seen at later time points, indicating extended blood circulation and enhanced tumor accumulation of the liposomal nanoparticles.

Fluorescence signal in the blood was monitored to estimate plasma clearance kinetics of DiR-labeled liposomal nanoparticles (Figure 6B). DiRL has the shortest blood circulation as its fluorescence signal decayed quickly and vanished entirely within 24 h. Its half-life ($t_{1/2}$) was only 4.0 h. LM-DiRL was found to have the most extended blood circulation with a half-life of 20.2 h. At 48 h, 35% of the administered LM-DiRL still stayed in the blood. Compared to cell membrane-devoid DiRL, the presence of leukocyte membrane in LM-DiRL extended the half-life, suggesting that leukocyte membrane camouflage helps LM-DiRL escape from the recognition and subsequent uptake by the MPS, presumably due to the presence of self-tolerance proteins on the leukocyte membrane as reported.^{14-16, 53} In contrast, the presence of tumor cell membrane in TM-DiRL did not significantly alter the half-life although the signal was detected up to 48 h. However, despite the presence of tumor

cell membrane, LTM-DiRL obtained a moderately extended plasma half-life of 8.1 h with the aid of the leukocyte membrane.

At 48 h post-administration, the major organs were harvested and imaged (Figure 6C). The prominent fluorescent signal of DiRL was seen in the liver and spleen. In contrast, TM-DiRL and leutosome (LTM-DiRL) show strong fluorescent signals in the tumors. DiR accumulation within various tissues was determined and is as shown in Figure 6D, tumor accumulation of LTM-DiRL increased 9.3-fold to $79.1 \pm 6.6\%$ ID per gram of tumor ($P < 0.01$) compared with the control DiRL ($8.5 \pm 3.4\%$). Although significantly lower than leutosome, tumor accumulation of LM-DiRL and TM-DiRL was 2.7-fold ($P < 0.05$) and 4.4-fold ($P < 0.01$) higher than that of DiRL, respectively. In contrast to enhanced accumulation in the tumors, significantly lower accumulation was noted in liver and spleen (in the range of 40%–63% of that of DiRL) as well as in lung (an average of 29% reduced lung accumulation vs. that of DiRL) when LM-DiRL, TM-DiRL or leutosome (LTM-DiRL) were used indicating tumor-specific delivery. The detailed contents about %ID per gram tissue and %ID per tissue were listed in the Table S1. Tumor cryosections were analyzed, and colocalization of YFP-expressing HN12 cells (green) and DiR-labeled liposomal nanoparticles (red) demonstrates selective uptake of DiR-labeled liposomal nanoparticles by HN12 cells (Figure 6E). Enhanced tumor accumulation of LM-DiRL is attributed to its extended blood circulation while TM-DiRL obtains high tumor accumulation by active targeting mechanism, in which homotypic binding interaction with the source tumor plays a crucial role.^{18, 19, 24} The biodistribution and plasma clearance kinetics data suggest that LTM-DiRL mimics not only the innate leukocytes to circulate in the blood for an extended period but also tumor cells to achieve active homotypic tumor targeting. We also prepared leutosome composed of membrane fractions of B16 melanoma cells and leukocytes (J774A.1). We observed its prominent accumulation within the B16 tumor rather than in the other organs (Figure S10), hence supporting the generalization of the approach.

The *in vivo* antitumor effects of PTX formulations were further assessed. Tumor-bearing mice received 6 doses 4 days apart via tail-vein intravenous administration. The tumor volume and body weight of mice were recorded to evaluate general antitumor effects (Figure 7A and 7B). The mice and tumor after different treatments were visualized at the endpoint (Figure 7C and 7D). The tumor sections from mice in each group were stained for tissue-level apoptosis detection (Figure 7E). Among different treatments, three different cell membranes camouflaged PTX-loaded liposomal nanoparticles showed the superior antitumor effect compared with others. Of note important, LTM-PTXL showed the strongest antitumor efficacy verified by slowest tumor growth rate, the smallest tumor size, and the most extensive cell apoptosis. Additionally, biosafety tests (Figure S11) including the levels of inflammatory cytokines (IL-1 β and TNF- α), hepatic function indicators (AST and ALT) and renal function indicators (BUN and CREA) showed that all the test formulations are biocompatible only except for Taxol, which caused significant toxicity to the liver and induced inflammatory reaction as evidenced by a 1.7-fold increase in both ALT and AST, a 2.2-fold increase in IL-1 β , and a 2.4-fold increase in TNF- α .

A number of nanoparticles-based drug delivery systems have been developed for cancer treatment and claimed varying degrees of success in extending the half-lives of drugs,

improving therapeutic index and reducing adverse effects as well as enhancing tumor targeting.^{54–56} However, nanoparticles functionalized with tumor-specific ligands are usually inadequate to achieve satisfactory tumor homing owing to the complex tumor microenvironment, incompetent ligand-receptor binding and insignificant EPR effect. Additionally, it poses a high risk of losing bioactivities of targeting ligands during chemical functionalization.^{52, 57} In parallel to synthetic tumor-targeting nanomedicine development, cellular vehicles have been actively exploited to deliver anticancer drugs.^{58, 59} The tumor microenvironment is a complex and dynamic network often associated with obvious inflammation.^{60, 61} Leukocytes have the intrinsic ability to home to the tumor in response to the persistent inflammation, hypoxia, and the elevated levels of mediators (such as cytokines, chemokines, matrix remodeling proteases and reactive oxygen species).^{62–64} Hijacking cells to deliver drugs via nanoparticles immobilized to the cell membrane was investigated by a few other research groups and us.^{32, 65–67} Recently tumor cell membrane-camouflaged nanoparticles have been developed on the basis of homotypic (between tumor cells) adhesive interactions, a critical event in the metastasis cascade.^{68, 69} They represent a new way to use cells and nanoparticles.

Although tumor cells may tone down the expression of tumor-specific antigens on the surface to mitigate immune detection,^{20, 70, 71} we found liposomal nanoparticles camouflaged with tumor cell membrane alone still caused a significant uptake by the MPS and were quickly cleared from blood circulation. In our work, incorporating leukocyte membrane along with tumor cell membrane extended the blood circulation and further enhanced the tumor homing of the nanoparticles. The leukocyte membrane plays an integral role in reducing the elimination of leutosome by the MPS and extending its circulation in the blood while the composite tumor cell membrane helps it actively accumulate in the homologous tumor via homotypic tumor binding property. Its strong *in vivo* antitumor effect substantiated the utility of the composite cell membrane camouflage to promote solid tumor homing. A recent meta-analysis surveying the literature from the past 10 years reveals that tumor accumulation of nanoparticles in rodent models via active targeting ranged from 0.02 to 45.8% ID/g with a median of 4.60% ID/g.⁷² Our liposomal vehicle camouflaged with tumor cell membrane only (TM-L) achieved a tumor accumulation of $37.3 \pm 6.4\%$ ID/g, making it rank among the highly tumor-selective formulations. We also noticed that it worked as efficiently as the recently reported tumor cell membrane-camouflaged nanoparticles in enhancing tumor accumulation of the camouflaged nanoparticles.^{73, 74} In striking contrast, the accumulation of leutosome in the tumor reached approximately 80% ID/g, 1.7-fold higher than the highest reported in the literature. Empowered with a high drug loading capacity, composite leukocyte and tumor cell membrane has proven more efficient than tumor cell membrane alone in directing camouflaged nanoparticles to the tumor. While the promising data are presented here, some questions have yet to be addressed including immune response to the camouflaged nanoparticle in connection with optimization of leukocyte and tumor cell membrane chimeric proportions for further reduced accumulation in the MPS organs and improved cancer therapy outcomes. To this end, it is of particular interest to examine this platform for its cancer immunotherapeutic activity in our future studies. The feasibility of using autologous tumor cells from patients to develop nanoformulations for personalized cancer medicine remains to be determined.

In conclusion, leukocyte membrane and tumor cell membrane can be simultaneously fused into liposomal nanoparticles to form leutosome, a composite membrane-camouflaged biomimetic nanoplatform. We confirmed that leutosome exhibited original surface biomarkers of both cell types. Leutosome was thoroughly characterized and compared with liposomal nanoparticles made of external phospholipids only or in a combination of the cell membrane of a single cell type *in vitro* and *in vivo*. In a leukocyte-tumor cell co-culture model, we observed that leutosome was taken up selectively by tumor cells while its uptake by leukocytes was reduced to a minimal level. Leutosome was found to have high drug loading capacity for PTX, and the PTX-encapsulating leutosome had reduced IC50. The *in vivo* studies conducted on a mouse xenograft model of head and neck cancer revealed that presence of the plasma membranes of leukocytes as well as tumor cells conferred prolonged blood circulation and significantly enhanced tumor accumulation of the camouflaged nanoparticles. The composite leukocyte and tumor cell membrane-camouflaged nanoparticles are superior to tumor cell membrane alone-camouflaged nanoparticles for tumor homing. Furthermore, leutosome strongly inhibits tumor growth *in vivo* during the treatment window and does not cause systemic toxicity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

This work was supported, in part, by the National Science Foundation (CAREER Award CBET0954957) (HY), VCU's CTSA (UL1TR000058 from the National Institutes of Health's National Center for Advancing Translational Science) and the Center for Clinical and Translational Research (CCTR) Endowment Fund of the Virginia Commonwealth University (HY and SG) as well as VA MERIT award (IO1 BX002297) (SG). HH was supported by a post-doctoral fellowship from the American Heart Association (17POST33660608). Microscopy was performed at the VCU Microscopy Facility, supported, in part, by funding from NIH-NCI Cancer Center Support Grant P30 CA016059. All authors have read the journal's policy on disclosure of potential conflicts of interest and have none to declare. All authors have read the journal's authorship agreement and the manuscript has been reviewed by and approved by all named authors.

REFERENCES

1. Shi J; Kantoff PW; Wooster R; Farokhzad OC *Nat. Rev. Cancer* 2017, 17 (1), 20. [PubMed: 27834398]
2. Björnalm M; Thurecht KJ; Michael M; Scott AM; Caruso F *ACS Nano* 2017, 11 (10), 9594–9613. [PubMed: 28926225]
3. Ferrari M *Nat. Rev. Cancer* 2005, 5, 161. [PubMed: 15738981]
4. Blanco E; Shen H; Ferrari M *Nat. Biotechnol* 2015, 33, 941. [PubMed: 26348965]
5. Kroll AV; Fang RH; Zhang L *Bioconjugate Chem* 2016, 28 (1), 23–32.
6. Luk BT; Zhang LJ *Controlled Release* 2015, 220, 600–607.
7. Gou M; Qu X; Zhu W; Xiang M; Yang J; Zhang K; Wei Y; Chen S *Nat. Commun.* 2014, 5, 3774. [PubMed: 24805923]
8. Hu C-MJ; Fang RH; Copp J; Luk BT; Zhang L *Nat. Nanotechnol* 2013, 8 (5), 336–340. [PubMed: 23584215]
9. Zhou H; Fan Z; Lemons PK; Cheng H *Theranostics* 2016, 6 (7), 1012. [PubMed: 27217834]
10. Hu C-MJ; Fang RH; Luk BT; Zhang L *Nat. Nanotechnol* 2013, 8 (12), 933–938. [PubMed: 24292514]

11. Hu C-MJ; Fang RH; Wang K-C; Luk BT; Thamphiwatana S; Dehaini D; Nguyen P; Angsantikul P; Wen CH; Kroll AV; et al. *Nature* 2015, 526 (7571), 118. [PubMed: 26374997]
12. He Y, Li R, Liang J ; Zhu Y; Zhang S; Zheng Z; Qin J; Pang Z; Wang J *Nano Res* 2018, <https://doi.org/10.1007/s12274-018-2126-5>.
13. Xuan M; Shao J; Dai L; He Q; Li J *Adv. Healthcare Mater* 2015, 4 (11), 1645–1652.
14. Parodi A; Quattrocchi N; Van De Ven AL; Chiappini C; Evangelopoulos M; Martinez JO; Brown BS; Khaled SZ; Yazdi IK; Enzo MV *Nat. Nanotechnol* 2013, 8 (1), 61–68. [PubMed: 23241654]
15. Molinaro R; Corbo C; Martinez JO; Taraballi F; Evangelopoulos M; Minardi S; Yazdi IK; Zhao P; De Rosa E; Sherman M *Nat. Mater* 2016, 15 (9), 1037. [PubMed: 27213956]
16. Palomba R; Parodi A; Evangelopoulos M; Acciardo S; Corbo C; De Rosa E; Yazdi I; Scaria S; Molinaro R; Furman NT; et al. *Sci. Rep* 2016, 6, 1–6. [PubMed: 28442746]
17. Cao H; Dan Z; He X; Zhang Z; Yu H; Yin Q; Li Y *ACS Nano* 2016, 10 (8), 7738–7748. [PubMed: 27454827]
18. Chen Z; Zhao P; Luo Z; Zheng M; Tian H; Gong P; Gao G; Pan H; Liu L; Ma A; et al. *ACS Nano* 2016, 10 (11), 10049–10057. [PubMed: 27934074]
19. Li S-Y; Cheng H; Qiu W-X; Zhang L; Wan S-S; Zeng J-Y; Zhang X-Z *Biomaterials* 2017, 142, 149–161. [PubMed: 28735175]
20. Fang RH; Hu C-MJ; Luk BT; Gao W; Copp JA; Tai Y; O'Connor DE; Zhang L *Nano Lett* 2014, 14 (4), 2181–2188. [PubMed: 24673373]
21. Sun H; Su J; Meng Q; Yin Q; Chen L; Gu W; Zhang P; Zhang Z; Yu H; Wang S; et al. *Adv. Mater* 2016, 28 (43), 9581–9588. [PubMed: 27628433]
22. Zhu J-Y; Zheng D-W; Zhang M-K; Yu W-Y; Qiu W-X; Hu J-J; Feng J; Zhang X-Z *Nano Lett* 2016, 16 (9), 5895–5901. [PubMed: 27513184]
23. Rao L; Bu LL; Cai B; Xu JH; Li A; Zhang WF; Sun ZJ; Guo SS; Liu W; Wang TH; et al. *Adv. Mater* 2016, 28 (18), 3460–3466. [PubMed: 26970518]
24. Sun H; Su J; Meng Q; Yin Q; Chen L; Gu W; Zhang Z; Yu H; Zhang P; Wang S; et al. *Adv. Funct. Mater* 2017, 27, 1604300.
25. Cheng H; Zhu JY; Li SY; Zeng JY; Lei Q; Chen KW; Zhang C; Zhang XZ *Adv. Funct. Mater* 2016, 26 (43), 7847–7860.
26. Tan S; Wu T; Zhang D; Zhang Z *Theranostics* 2015, 5 (8), 863. [PubMed: 26000058]
27. Zhai Y; Su J; Ran W; Zhang P; Yin Q; Zhang Z; Yu H; Li Y *Theranostics* 2017, 7 (10), 2575. [PubMed: 28819448]
28. Dehaini D; Fang RH; Zhang L *Bioeng. Transl. Med* 2016, 1 (1), 30–46. [PubMed: 29313005]
29. Rüegg CJ *Leukoc. Biol* 2006, 80 (4), 682–684.
30. Dong X; Chu D; Wang Z *Theranostics* 2017, 7 (3), 751. [PubMed: 28255364]
31. Xue J; Zhao Z; Zhang L; Xue L; Shen S; Wen Y; Wei Z; Wang L; Kong L; Sun H *Nat. Nanotechnol* 2017, 12, 692–700. [PubMed: 28650441]
32. Holden CA; Yuan Q; Yeudall WA; Lebman DA; Yang H *Int. J. Nanomed* 2010, 5, 25–36.
33. Xu L; Zolotarskaya OY; Yeudall WA; Yang H *Adv. Healthcare Mater* 2014, 3 (9), 1430–1438.
34. Mitchell MJ; King MR *Expert Opin. Drug Delivery* 2015, 12 (3), 375–92.
35. Miller MA; Zheng YR; Gadde S; Pfirschke C; Zope H; Engblom C; Kohler RH; Iwamoto Y; Yang KS; Askevold B; et al. *Nat. Commun* 2015, 6, 8692. [PubMed: 26503691]
36. Molinaro R; Corbo C; Martinez JO; Taraballi F; Evangelopoulos M; Minardi S; Yazdi IK; Zhao P; De Rosa E; Sherman M *Nat. Mater* 2016, 15 (9), 1037. [PubMed: 27213956]
37. Palomba R; Parodi A; Evangelopoulos M; Acciardo S; Corbo C; De Rosa E; Yazdi I; Scaria S; Molinaro R; Furman NT *Sci. Rep* 2016, 6, 34422. [PubMed: 27703233]
38. Zhang Y; Cai K; Li C; Guo Q; Chen Q; He X; Liu L; Zhang Y; Lu Y; Chen X *Nano Lett* 2018, 18(3), 1908–1915. [PubMed: 29473753]
39. Krishnamurthy S; Gnanasammandhan M; Xie C; Huang K; Cui M; Chan J *Nanoscale* 2016, 8 (13), 6981–6985. [PubMed: 26975904]
40. Wang J; Dzuricky M; Chilkoti A *Nano Lett* 2017, 17 (10), 5995–6005. [PubMed: 28853896]

41. Chen X; Wong R; Khalidov I; Wang AY; Leelawattanachai J; Wang Y; Jin MM *Biomaterials* 2011, 32 (30), 7651–61. [PubMed: 21783245]
42. Dong X; Chu D; Wang Z *Theranostics* 2017, 7 (3), 751–763. [PubMed: 28255364]
43. Glinsky VV; Glinsky GV; Glinskii OV; Huxley VH; Turk JR; Mossine VV; Deutscher SL; Pienta KJ; Quinn TP *Cancer Res* 2003, 63 (13), 3805–3811. [PubMed: 12839977]
44. Khaldoyanidi SK; Glinsky VV; Sikora L; Glinskii AB; Mossine VV; Quinn TP; Glinsky GV; Sriramarao PJ *Biol. Chem* 2003, 278 (6), 4127–4134.
45. Cheung AS; Koshy ST; Stafford AG; Bastings M; Mooney DJ *Small* 2016, 12 (17), 2321–2333. [PubMed: 26953489]
46. Kroll AV; Fang RH; Jiang Y; Zhou J; Wei X; Yu CL; Gao J; Luk BT; Dehaini D; Gao W; et al. *Adv. Mater* 2017, 29 (47), 1703969.
47. Parodi A; Quattrocchi N; Van De Ven AL; Chiappini C; Evangelopoulos M; Martinez JO; Brown BS; Khaled SZ; Yazdi IK; Enzo MV *Nat. Nanotechnol* 2013, 8 (1), 61. [PubMed: 23241654]
48. Dehaini D; Wei X; Fang RH; Masson S; Angsantikul P; Luk BT; Zhang Y; Ying M; Jiang Y; Kroll AV; et al. *Adv. Mater* 2017, 29 (16), 1606209.
49. Jia J; Xiao Y; Liu J; Zhang W; He H; Chen L; Zhang MJ *Pharm. Sci* 2012, 101 (8), 2900–2908.
50. Zhao H; Wang J-C; Sun Q-S; Luo C-L; Zhang QJ *Drug Targeting* 2009, 17 (1), 10–18.
51. Gustafson HH; Holt-Casper D; Grainger DW; Ghandehari H *Nano Today* 2015, 10 (4), 487–510. [PubMed: 26640510]
52. Blanco E; Shen H; Ferrari M *Nat. Biotechnol* 2015, 33 (9), 941–951. [PubMed: 26348965]
53. Evangelopoulos M; Parodi A; Martinez JO; Yazdi IK; Cevenini A; van de Ven AL; Quattrocchi N; Boada C; Taghipour N; Corbo C *Biomaterials* 2016, 82, 168–177. [PubMed: 26761780]
54. Shi J; Kantoff PW; Wooster R; Farokhzad OC *Nat. Rev. Cancer* 2017, 17 (1), 20. [PubMed: 27834398]
55. He H; Ghosh S; Yang HJ *Controlled Release* 2017, 247, 106–126.
56. Sun T; Zhang YS; Pang B; Hyun DC; Yang M; Xia Y *Angew. Chem. Int. Ed. Engl* 2014, 53 (46), 12320–12364. [PubMed: 25294565]
57. Chen G; Roy I; Yang C; Prasad PN *Chem. Rev* 2016, 116 (5), 2826–2885. [PubMed: 26799741]
58. Danhier F; Feron O; Préat VJ *Controlled Release* 2010, 148 (2), 135–146.
59. Khawar IA; Kim JH; Kuh H-JJ *Controlled Release* 2015, 201, 78–89.
60. Coussens LM; Werb Z *Nature* 2002, 420 (6917), 860. [PubMed: 12490959]
61. Gajewski TF; Schreiber H; Fu Y-X *Nat. Immunol* 2013, 14 (10), 1014–1022. [PubMed: 24048123]
62. Balkwill F *Nat. Rev. Cancer* 2004, 4 (7), 540–550. [PubMed: 15229479]
63. Whiteside T *Oncogene* 2008, 27 (45), 5904. [PubMed: 18836471]
64. Mantovani A; Allavena P; Sica A; Balkwill F *Nature* 2008, 454 (7203), 436–444. [PubMed: 18650914]
65. Xu L; Zolotarskaya OY; Yeudall WA; Yang H *Adv. Healthcare Mater* 2014, 3 (9), 1430–8.
66. Stephan MT; Moon JJ; Um SH; Bershteyn A; Irvine DJ *Nat. Med* 2010, 16 (9), 1035–41. [PubMed: 20711198]
67. Su Y; Xie Z; Kim GB; Dong C; Yang J *ACS Biomater. Sci. Eng* 2015, 1 (4), 201–217. [PubMed: 25984572]
68. Wei SC; Yang J *Trends Cell Biol* 2016, 26 (2), 111–120. [PubMed: 26508691]
69. Zamir E; Geiger BJ *Cell Sci* 2001, 114 (Pt 20), 3583–90.
70. Yoshihama S; Roszik J; Downs I; Meissner TB; Vijayan S; Chapuy B; Sidiq T; Shipp MA; Lizée GA; Kobayashi KS *Proc. Natl. Acad. Sci. U. S. A* 2016, 113 (21), 5999–6004. [PubMed: 27162338]
71. Drake CG; Jaffee E; Pardoll DM *Adv. Immunol* 2006, 90, 51–81. [PubMed: 16730261]
72. Björnmalm M; Thurecht KJ; Michael M; Scott AM; Caruso F *ACS Nano* 2017, 11 (10), 9594–9613. [PubMed: 28926225]
73. Sun H; Su J; Meng Q; Yin Q; Chen L; Gu W; Zhang P; Zhang Z; Yu H; Wang S; et al. *Adv. Mater* 2016, 28 (43), 9581–9588. [PubMed: 27628433]

74. Rao L; Bu LL; Cai B; Xu JH; Li A; Zhang WF; Sun ZJ; Guo SS; Liu W; Wang TH; et al. Adv. Mater 2016, 28 (18), 3460–6. [PubMed: 26970518]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 1.

Verification and characterizations of incorporating two cell membranes into leutosome. (A) Visualization of extracted dual dyes-labeled composite cells membrane before and after extrusion under confocal microscopy and TEM, respectively. DiO-labeled leukocyte membranes are shown as green fluorescence and DiD-labeled tumor cell membranes are shown as red fluorescence. Two types of cell membranes in microscale well fused together to form the nanoscale leutosome after extrusion. (B) Intracellular colocalization of composite nanoscale dual dyes-labeled cells membrane-derived vesicle in the source tumor

cell HN12 after 6 h incubation. DAPI channel is for nuclei, DiO channel for leukocytes membrane and DiD channel for tumor cells.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 2. Characterization of PTX-loaded liposomal nanoparticles.

(A) Membrane-specific and intracellular markers characterization by Western analysis for lysates from leukocytes and tumor cells (1), cell membranes extracted from those two cells (2), empty leutosome (3) and PTX-loaded leutosome (4). (B) TEM images. (C) PTX encapsulation efficiency as a function of drug-to-lipid mass ratio, (D) PTX loading content (mole %) of formulations prepared at various drug-to-lipid mass ratio. (E) Particle size change and (F) zeta potential change at 4 °C for 1 week-storage. (G) *In vitro* PTX release behavior from different PTX liposomes in PBS at 37 °C. NS, not significant; ** indicates $p < 0.01$, and *** indicates $p < 0.001$ (n=3).

Figure 3.

Intracellular uptake of different cell membrane-camouflaged liposomal nanoparticles in the co-culture model established using J774A.1 leukocytes and YFP-expressing HN12 tumor cells. The cells were imaged after 6 h incubation and are shown in Figure 3A. The cells with both green signal and blue signal are YFP-expressing HN12, while the cells only with the blue signal are J774A.1. The red signal comes from the different DiI-labeled liposomal nanoparticles. Flow cytometric quantitative analysis results are shown in Figure 3B. ** indicates $p < 0.01$.

Figure 4.

In vitro cytotoxicity of (A) empty liposomal nanoparticles with and without different cell membrane camouflage, and different PTX-loaded liposomal nanoparticles (B) at various concentrations on HN12 cells after 24 h incubation. **indicates $p < 0.01$ (n=6).

Figure 5.

Leukocyte membrane helps reduce the uptake of nanoparticles by monocytes and neutrophils in the blood. * indicates $p < 0.05$ and ** indicates $p < 0.01$ ($n = 4$).

Figure 6.

In vivo distribution and pharmacokinetics of liposomal nanoparticles (with and without cell membrane camouflaging). (A) *In vivo* fluorescence imaging at different time points. (B) *In vivo* blood clearance of the liposomal nanoparticles as measured by the fluorescence intensity. (C) *Ex vivo* tissue distribution in the main organs at 48 h post-i.v. administration. (D) Quantitative analysis of fluorescence accumulation in the main organs. (E) Representative fluorescent images of the tumor sections. ** indicates $p < 0.01$ and *** indicates $p < 0.001$ ($n = 4$).

Figure 7. Leutosome significantly suppressed tumor growth. (A) Relative tumor volume growth and (B) body weight changes were monitored over the treatments. Photographs of HN12 tumor-bearing mice (C), the excised tumors (D) and tumor tissue apoptosis (E) were recorded after the various treatments. NS, not significant; ** indicates $p < 0.01$, and *** indicates $p < 0.001$ (n=6).

Scheme 1.

Schematic presentation of composite leukocyte and tumor cell membrane-camouflaged liposome (leutosome) as a carrier for paclitaxel (LTM-PTXL) and potential application in cancer treatment and diagnosis. Both leukocyte and tumor cell membranes are extracted and applied to hydrate the thin film containing payloads (paclitaxel or fluorescent dyes) to obtain the resulting leutosome via sonication and extrusion. The resulting LTM-PTXL would integrate each original cell's biofunctions (leukocyte's avoidance of the uptake by the MPS, long residency in blood and recruitment to the tumor microenvironment, and tumor cell's

homotypic tumor targeting) into one entity, thus prolonging the blood circulation, increasing the tumor accumulation and improving antitumor efficacy.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript