Title
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Permalink
https://escholarship.org/uc/item/1t86b832

Journal
Scientific reports, 10(1)

ISSN
2045-2322

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Publication Date
2020-07-09

DOI
10.1038/s41598-020-68190-0

Peer reviewed
Polymerized human hemoglobin facilitated modulation of tumor oxygenation is dependent on tumor oxygenation status and oxygen affinity of the hemoglobin-based oxygen carrier

Donald A. Belcher\(^1,3\), Alfredo Lucas\(^2,3\), Pedro Cabrales\(^2\) & Andre F. Palmer\(^1\)*

Administration of hemoglobin-based oxygen carriers (HBOCs) into the systemic circulation is a potential strategy to relieve solid tumor hypoxia in order to increase the effectiveness of chemotherapeutics. Previous computational analysis indicated that the oxygen (\(O_2\)) status of the tumor and HBOC \(O_2\) affinity may play a role in increased \(O_2\) delivery to the tumor. However, no study has experimentally investigated how low- and high-affinity HBOCs would perform in normoxic and hypoxic tumors. In this study, we examined how the HBOC, polymerized human hemoglobin (PolyhHb), in the relaxed (R) or tense (T) quaternary state modulates \(O_2\) delivery to hypoxic (FME) and normoxic (LOX) human melanoma xenografts in a murine window chamber model. We examined microcirculatory fluid flow via video shearing optical microscopy, and \(O_2\) distributions via phosphorescence quenching microscopy. Additionally, we examined how weekly infusion of a 20% top-load dose of PolyhHb influences growth rate, vascularization, and regional blood flow in the FME and LOX tumor xenografts. Infusion of low-affinity T-state PolyhHb led to increased tissue oxygenation, decreased blood flow, decreased tumor growth, and decreased vascularization in hypoxic tumors. However, infusion of both T-state and R-state PolyhHbs led to worse outcomes in normoxic tumors. Of particular concern was the high-affinity R-state PolyhHb, which led to no improvement in hypoxic tumors and significantly worsened outcomes in normoxic tumors. Taken together, the results of this study indicate that the tumor \(O_2\) status is a primary determinant of the potency and outcomes of infused PolyhHb.

Unregulated angiogenesis and rapid cell proliferation in the tumor microenvironment result in decreased blood flow and oxygen (\(O_2\)) delivery\(^4\). Under these conditions, cancer cells adapt to the hypoxic environment via activation of hypoxia-inducible factors, HIF-1 and HIF-2\(^2\). These adaptations to chronic hypoxia are associated with metabolic reprogramming, angiogenesis, epithelial-mesenchymal transition, metastasis, and resistance to radiation and chemotherapy\(^3,4\). Furthermore, many forms of cancer therapy require reactive oxygen species (ROS) to promote tumor suppression\(^5\). Thus, increasing \(O_2\) delivery to solid tumors is a promising target for cancer therapy.

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Due to their ability to modulate O₂ delivery from the circulatory system, hemoglobin-based oxygen carriers (HBOCs) are promising O₂ therapeutics that may increase the effectiveness of chemotherapy. However, HBOCs are still not clinically approved despite decades of research. The elevated renal toxicity and hypertension associated with previous generations of commercial HBOCs have mainly been attributed to this delay in development. Current improvements in reactor design and product purification are now able to exclude the low molecular weight (MW) HBOC fractions (≤ 250 kDa) that contributed to the toxicity of early generation HBOCs.

The chemical modifications that are necessary to make HBOCs safe for infusion typically alter the HBOC O₂ affinity. For example, conjugating polyethylene glycol to the surface of hemoglobin (Hb) results in an increased O₂ affinity (P₅₀: 5–6 mm Hg) compared to unmodified Hb. Alternatively, commercial polymerized Hbs (PolyHbs) prepared via glutaraldehyde or O-raffinose crosslinking in the tense (T) quaternary state all have significantly lower O₂ affinity (P₅₀: 30–40 mm Hg) compared to unmodified Hb. Recently, we demonstrated that the O₂ affinity of PolyHbs could be controlled by polymerizing the Hb under fully oxygenated or deoxygenated conditions. By fully oxygenating the Hb during polymerization, the PolyHb is effectively locked into the high O₂ affinity, relaxed quaternary state (R-state). Alternatively, polymerizing the Hb while deoxygenated locks the PolyHb into the low O₂ affinity, T-state.

Previously, we computationally determined that alterations in the O₂ affinity of the HBOC impact how much O₂ an HBOC will supply to the surrounding tumor tissue. From the results of these studies, we anticipate that R-state PolyHb and T-state PolyHb should increase O₂ delivery to hypoxic tissue. Whereas in normoxic tissue, T-state PolyHb should increase oxygenation. To date, no study has examined how infusing an HBOC may impact the growth and vascularization of hypoxic and normoxic tumors. Previous studies only examined hypoxic tumors or a single class of HBOC. Understanding how both normoxic and hypoxic tumors respond to different modes of enhanced O₂ delivery is vital to defining how these materials would be applied clinically.

In this work, we prepared high MW polymerized human Hbs (PolyHbs) in either the T- or R-state. We then observed how each of these materials modulate O₂ transport within hypoxic (FME) and normoxic (LOX) human melanoma xenografts. By using the LOX melanoma cell line, we are able to generate normoxic tumors to compare with the hypoxic tumors. By using phosophorecence quenching microscopy (PQM) on the microcirculatory environment observed within window chamber models, we explored how PolyHbs modulate blood flow and O₂ transport in vivo. Additionally, we examined how weekly infusions of PolyHb impact tumor growth and vascularization. With these results, we are better able to define how the oxygenation status of the tumor and the O₂ affinity of an HBOC will impact modulation of O₂ delivery.

**Results**

**Biophysical properties of PolyHbs.** The resulting properties of the PolyHbs used in this study are shown in Fig. 1. The metHb levels of the resulting solutions were 5.8 ± 0.8% for 35:1 T-state PolyHb and 4.2 ± 0.9% for 30:1 R-state PolyHb. The concentration of both solutions was maintained at 100 ± 1.5 mg/mL. Polymerization under fully oxygenated conditions resulted in a significant increase in O₂ affinity compared to unmodified Hb. After polymerization under completely deoxygenated conditions significantly decreased O₂ affinity. After polymerization, the rate of O₂ release is significantly slower when compared to unmodified Hb. The 30:1 R-state PolyHb (D = 38 ± 5.3 nm) was smaller than 35:1 T-state PolyHb (D = 63.7 ± 7.3 nm). Despite being larger on average, 35:1 T-state PolyHb contained significantly more low MW species (0th, 1st order) compared to 30:1 T-state PolyHb.

**Hematological and blood gas parameters.** Changes in hematological and blood gas parameters are displayed in Table 1. There were no differences in systemic blood parameters at baseline compared to healthy mice (no implanted tumor). Infusion of 35:1 T-state and 30:1 R-state PolyHb significantly reduced the hematocrit. There was no significant difference between either the hematocrit or PolyHb in the plasma after infusion of either 35:1 T-state or 30:1 R-state PolyHb. Infusion of both 35:1 T-state PolyHb and 30:1 R-state PolyHb resulted in significant increases in MAP. In mice implanted with FME tumors, infusion of 30:1 R-state PolyHb led to a significant decrease in HR compared to the Baseline. In contrast, infusion 35:1 T-state PolyHb led to significant increases in average tissue pO₂ in both tumors.

**Tissue O₂ tension.** Changes in tissue O₂ tension in both hypoxic (FME) and normoxic (LOX) tumors are depicted in Fig. 2. The average tissue pO₂ in hypoxic (FME) tumors was significantly lower than the average tissue pO₂ in normoxic (LOX) tumors and host tissue. Infusion of 30:1 R-state PolyHb significantly decreased tissue oxygenation in both normoxic (LOX) and hypoxic (FME) tumors compared to baseline and following infusion of 35:1 T-state PolyHb. In contrast, infusion 35:1 T-state PolyHb led to significant increases in average tissue pO₂ in both tumors.

**Microhemodynamics.** Changes in the arteriolar and venular blood flow in both hypoxic (FME) and normoxic (LOX) tumors are depicted in Fig. 3A, B. Vessel diameters were separated into two groups for this analysis: one group with diameters less than 30 μm and another group with diameters greater than 30 μm. In hypoxic tumors, infusion of 30:1 R-state PolyHb led to a significant reduction in the volumetric flow rate in small diameter (D_{avg} < 30 μm) venules. Infusion of 35:1 T-state PolyHb led to significant increases in the flow...
rate in large diameter ($D_{ves} > 30$ μm) venules. In normoxic tumors, infusion of both PolyhHb species led to significant increases in tumor perfusion in all blood vessels. In general, blood flow velocity in normoxic tumors was significantly higher than blood flow in hypoxic tumors.

Figure 1. Biophysical properties of unmodified hHb, 35:1 T-state PolyhHb, and 30:1 R-state PolyhHb. (A) OECs for PolyhHb and hHb. (B) Comparison of the kinetic time course for deoxygenation in the presence of 1.5 mg/mL sodium dithionite for hHb, 30:1 R-state PolyhHb, and T-state PolyhHb. For OECs (A), The shaded region represents the 95% confidence interval for each quaternary state with three runs per sample. For deoxygenation (B), the experimental data shows an average of 10–15 kinetic traces. For deoxygenation, the reactions were monitored at 437.5 nm and 20°C in 0.1 M pH 7.4 PBS. Symbols represent experimental data, and corresponding lines of the same color represent curve fits. (C) Representative intensity distributions of the hydrodynamic diameter of 30:1 R-state and 35:1 T-state PolyhHb. (D) Normalized SEC intensity distributions of R-state and T-state PolyhHb compared to unmodified hHb. The shaded region represents the 95% confidence interval for the average of the produced PolyhHb. (E) Polymer order distribution for 35:1 T-state and 30:1 R-state PolyhHb. Polymer distribution was calculated on a percent by heme basis via analysis of the 413 nm absorbance wavelength. The corresponding approximate sizes of the polymer orders are shown below each group.
Table 1. Hematological and blood gas parameters for healthy animals and animals implanted with FME or LOX tumor infused with a 20% top-load dose of either 35:1 T-state PolyhHb or 30:1 R-state PolyhHb. †P < 0.05 compared to baseline. ‡P < 0.05 compared to 35:1 T-state PolyhHb (n = 6 mice).

| Parameter               | Healthy | FME human melanoma xenograft | LOX human melanoma xenograft |
|-------------------------|---------|------------------------------|------------------------------|
|                         | Baseline| 35:1 T-state PolyhHb | 30:1 R-state PolyhHb | Baseline | 35:1 T-state PolyhHb | 30:1 R-state PolyhHb |
| Hb (g/dL)               | 14.6 ± 0.2 | 14.6 ± 0.2† | 10.8 ± 0.4‡ | 11.3 ± 0.5† | 14.6 ± 0.2† | 10.9 ± 0.2‡ | 10.9 ± 0.2† |
| Plasma PolyhHb (g/dL)  | –       | 1.4 ± 0.1 | 1.3 ± 0.1   | –       | 1.3 ± 0.1   | 1.5 ± 0.1   |
| MAP (mmHg)              | 105 ± 7 | 104 ± 6† | 122 ± 4‡   | 126 ± 9† | 105 ± 8‡ | 128 ± 5† | 125 ± 9‡ |
| HR (beats/min)          | 517 ± 28 | 514 ± 26 | 492 ± 25   | 469 ± 28† | 518 ± 25 | 484 ± 27† | 496 ± 22 |
| PaO2 (mmHg)             | 34.7 ± 1.9 | 35.0 ± 2.2 | 35.1 ± 2.0 | 32.0 ± 1.3†‡ | 34.5 ± 1.6 | 34.9 ± 1.5 | 32.3 ± 2.5‡ |
| PaCO2 (mmHg)            | 7.29 ± 0.09 | 7.32 ± 0.10† | 7.38 ± 0.09† | 7.25 ± 0.12†‡ | 7.29 ± 0.07‡ | 7.35 ± 0.10† | 7.26 ± 0.10†‡ |
| pH                      | 7.29 ± 0.09 | 7.32 ± 0.10† | 7.38 ± 0.09† | 7.25 ± 0.12†‡ | 7.29 ± 0.07‡ | 7.35 ± 0.10† | 7.26 ± 0.10†‡ |

Figure 2. Average tissue pO2 and distribution of measured tissue pO2 in host tissue, and in hypoxic, and normoxic tumor xenografts as measured via phosphorescence quenching microscopy in the chamber window model. This figure shows the (A, C) average pO2s and (B, D) frequency of tissue pO2 for (A, B) hypoxic FME human melanoma and (C, D) normoxic LOX human melanoma tumor xenografts. Data is shown for the baseline and after infusion of 30:1 R-state PolyhHb and 35:1 T-state PolyhHb. †P < 0.05 compared to baseline. ‡P < 0.05 compared to 35:1 T-state PolyhHb (n = 5 mice).
Intravascular pO2 and O2 saturations. Changes in intravascular pO2, O2 saturation of Hb in RBCs, and O2 saturation of PolyhHbs are shown in Fig. 3C–E. The O2 saturation of Hb in RBCs was always significantly greater in the normoxic tumor compared to the hypoxic tumor. Infusion of 30:1 R-state PolyhHb led to significant reduction in blood O2 saturation in hypoxic tumors compared to both the control and infusion of 35:1 T-state PolyhHb. This is coupled with a corresponding significant (P < 0.05) decrease in O2 saturation of Hb in RBCs compared to the control and infusion of 35:1 T-state PolyhHb. Infusion of 35:1 T-state PolyhHb leads to a significant increase in intravascular pO2 in large diameter (D_ves > 30 µm) arterioles in the hypoxic tumor and

![Figure 3](https://doi.org/10.1038/s41598-020-68190-0)
large diameter \( (D_{ve} > 30 \, \mu m) \) venules in the normoxic tumor. Additionally, infusion of 35:1 T-state PolyhHb significantly \((P < 0.05)\) increased \(O_2\) saturation of Hb in RBCs in the arterioles of hypoxic tumors and the venules of normoxic tumors. As anticipated, the \(O_2\) saturation of 30:1 R-state PolyhHb was always significantly greater than the \(O_2\) saturation of 35:1 T-state PolyhHb.

**Oxygen extraction fraction (OEF).** The changes in the calculated plasma OEF, \(Hb\) in RBC OEF, PolyhHb OEF, and overall OEF in hypoxic (FME) and normoxic (LOX) tumors are displayed in Fig. 4. Infusion of 30:1 R-state PolyhHb significantly increased the amount of \(O_2\) extracted from plasma and RBCs in hypoxic (FME) tumors. Infusion of 35:1 T-state PolyhHb led to a significant reduction in the OEF from plasma and Hb in RBCs. The OEF from 35:1 T-state PolyhHb was significantly greater than the \(O_2\) extraction from 30:1 R-state PolyhHb. Infusion of 30:1 R-state PolyhHb decreased overall OEF in both hypoxic (FME) and normoxic (LOX) tumors. Infusion of 35:1 T-state PolyhHb led to significant reduction in overall OEF in LOX tumors; however, it did not lead to any significant changes in the OEF in FME tumors.

**Tumor growth.** In addition to examining how PolyhHb altered microvascular \(O_2\) transport in tumors, we also examined how a low volume weekly dose of 30:1 R-state PolyhHb and 35:1 T-state PolyhHb would impact tumors implanted into tissue. The effects of weekly 20% top-load doses of 30:1 R-state PolyhHb and 35:1 T-state PolyhHb on the volumes of FME and LOX tumor xenografts are shown in Fig. 5. The approximate rate of radial expansion was approximately 5.5 \(\mu m/h\) for FME (hypoxic) tumors and approximately 4.1 \(\mu m/h\) for LOX (normoxic) tumors. After top-load infusion of both T-state and R-state PolyhHb, there is a significant reduction in the growth of the hypoxic FME tumor. Infusion of 35:1 T-state PolyhHb resulted in a significant reduction in FME tumor growth (20%) compared to 30:1 R-state PolyhHb (9.6%).

**Tumor vasculature.** We were also interested in experimentally observing how periodic infusions of PolyhHb solutions would alter properties associated with microvascular mass transport. Regional blood flow (RBF) was analyzed with fluorescent microsphere perfusion, and microvascular density (MVD) was estimated via tissue histology. The results of these studies are shown in Fig. 5B, C. In hypoxic FME tumor xenografts, infusion of 35:1 T-state PolyhHb led to a significant decrease in both RBF and MVD. In normoxic LOX tumor xenografts, infusion of T-state PolyhHb led to a significant increase in RBF to the tumor compared to baseline.

**Discussion**

The principal finding of this study is that the \(O_2\) status of tumors has a strong effect on the effects of PolyhHb coadministration. These results may help explain some of the previous negative results that occurred in rhabdomyosarcomas\(^8\).

In this study, we investigated how infusion of PolyhHb influences microcirculatory fluid and \(O_2\) transport within a murine chamber window model. In general, infusion of 35:1 T-state PolyhHb led to an increase in tumor perfusion and \(O_2\) delivery. In contrast, infusion of 30:1 R-state PolyhHb decreased overall fluid and \(O_2\) transport. This can be attributed to two factors: (1) 30:1 R-state PolyhHb had extremely high \(O_2\) affinity, and (2) 30:1 R-state PolyhHb was slightly hypertensive. After infusion of 30:1 R-state PolyhHb there was a significant increase in MAP and \(PaO_2\) coupled with a decrease in \(PaCO_2\), HR, and pH. In comparison, infusion of 35:1 T-state PolyhHb...
primarily increased MAP. These increases in MAP is consistent with previous HBOCs containing low MW species\textsuperscript{36,37}.

In normoxic tumors, the hemodilution effect of PolyhHb infusion led to significant increases in tumor perfusion. Despite this increase in blood perfusion after the infusion of R-state PolyhHb, the average tissue \(pO_2\) decreased. This decrease is likely a result of the relatively low amount of \(O_2\) extracted from R-state PolyhHb (2.2 ± 0.1%), which failed to make up for the lack of \(O_2\) carrying capacity from the diluted blood. In comparison, 18.7 ± 4.7% of the total available \(O_2\) from T-state PolyhHb was extracted, which offset the \(O_2\) extraction from both Hb in RBCs and dissolved \(O_2\) in plasma.

In hypoxic tumors, the percentage of \(O_2\) extracted from 30:1 R-state PolyhHb only slightly increased (12.8 ± 1.8%) compared to the percentage of \(O_2\) extracted from 35:1 T-state PolyhHb (37.5 ± 2.6%). This slight increase in \(O_2\) extraction from R-state PolyhHb does not fully offset the \(O_2\) demand from \(O_2\) dissolved in plasma and Hb in RBCs. When the decrease in \(O_2\) supply from R-state PolyhHb is coupled with reduced blood flow resulting from vasoconstriction, overall \(O_2\) delivery is significantly reduced. This requirement is instead offset by significant increases in the \(O_2\) extracted by the dissolved \(O_2\) in the plasma and Hb in RBCs.

In this study, we found that the infusion of PolyhHb led to relatively minor changes in the growth rate of the hypoxic (FME) tumors. However, it appears that this low dosage was unable to replicate the significant reduction in tumor growth (40%) that was previously observed in a triple-negative breast cancer model\textsuperscript{20}. This is likely because the dose volume and dose frequency were too low to result in an appreciable effect on tumor growth. For this study, the PolyhHb was delivered weekly; however, previous studies of the pharmacokinetics of similar PolyHbs indicate that these PolyHbs have a half-life of only 24 h\textsuperscript{10}. Taking this into account, the tumors were only exposed to the \(O_2\) modulating effect of PolyhHb for only 25% of the week. Increasing the dosing frequency to once every 2 to 3 days may increase the relative effect.

Unfortunately, infusion of both the T-state and R-state PolyhHb solutions led to a significant increase in tumor growth for normoxic LOX tumors. Infusion of 30:1 R-state PolyhHb led to a 40% increase in tumor volume after the 14-day treatment regime. This is likely because both 35:1 T-state PolyhHb and 30:1 R-State PolyhHb
have higher O₂ affinity compared to mouse Hb in RBCs (P₅₀ = 42 mm Hg). Because of this relative increase in O₂ affinity, we anticipate that less O₂ may be delivered under normoxic conditions, which could decrease host cell survival in the tumor periphery. We anticipate that when applied to a model that more accurately represents human physiology, T-state PolyHb might decrease the tumor growth rate due to its lower O₂ affinity compared to human Hb in RBCs (P₅₀ = 26 mm Hg). However, we may also observe further increases in tumor growth due to the increased supply of O₂ to normoxic tumors.

Despite observing a growth delay after infusion of 30:1 R-state PolyHb, infusion of R-state PolyHb did not lead to significant decreases in RBF or MVD. This is likely because the low dose frequency and high O₂ affinity of R-state PolyHb were insufficient to trigger an anti-angiogenic response in FME tumors. Baseline values for RBF in hypoxic FME tumors [0.16 ± 0.02 mL/(min g)] and normoxic LOX tumors [0.15 ± 0.02 mL/(min g)] are at the upper range of the values measured for other tumors experimentally. In the hypoxic (FME) tumors, we observed decreases in MVD after delivery of 35:1 T-state PolyHb. This decrease in vessel formation indicates an increase in O₂ delivery. In contrast, we observed a significant increase in MVD after weekly infusions of 30:1 R-state PolyHb in normoxic (LOX) tumors. This is consistent with a decrease in O₂ delivery, which may lead to more aggressive tumor growth and increased angiogenesis. In fact, the MVD of the normoxic LOX tumor after infusion of 30:1 R-state PolyHb is remarkably similar to the measured MVD within the baseline hypoxic (FME) tumor. This further supports the notion that within the normoxic tumor, R-state PolyHb is not adequately delivering O₂, which is in agreement with microvascular simulations performed previously. Despite this, reduction in tumor growth has been previously observed after infusion of high O₂ affinity HBOCs. Therefore, this decrease in tumor growth may be due to other factors in the environment including production of reactive O₂ species (ROS) and nitric oxide (NO) scavenging. This faster rate of metHb formation will lead to the increased production of ROS which can induce oxidative injury to the tumor mass. This is especially important to consider given that HBOCs can scavenge NO and can oxidize and produce ROS species in vivo.

Future studies should investigate these mechanisms in more detail by directly observing changes in hypoxia inducible factors and downstream proteins when working with high O₂ affinity HBOCs.

Conclusions

The results from this study indicate that low-dose, infrequent infusions of R-state PolyHb is not suitable for oxygenating both hypoxic and normoxic melanomas. In general, treatment of normoxic tumors with either high- or low O₂ affinity PolyHbs aggravated tumor growth and angiogenesis. In contrast, T-state PolyHbs significantly increased O₂ supply to hypoxic tumors. These results encourage the use of low O₂ affinity PolyHbs with reduced cooperativity to hypoxic tumors. Additionally, this further emphasizes the need to fully characterize how different tumor types respond to modulating O₂ delivery with HBOCs.

Methods

Polymerized hemoglobin synthesis and analysis. Human Hb (hHb) used in these studies was first purified from human red blood cells (RBCs) as described previously. PolyHb was produced using methods described previously. In brief, the resulting hHb solution was polymerized with glutaraldehyde while fully oxygenated or deoxygenated to form either R-state or T-state PolyHb, respectively. The resulting PolyHb solutions were first clarified on a 0.2 μm hollow fiber filter. After clarification, the PolyHb solutions were diafiltered on a 100 kDa hollow fiber filter into a modified Ringer’s lactate buffer to remove the low MW PolyHb/hHb species.

The cyanomethemoglobin method was used to measure the Hb concentration and the metHb level of hHb/PolyHb solutions. The size distribution of PolyHb, by particle volume, was measured using dynamic light scattering (DLS) (Brookhaven Instrument Inc. BS-200M, Holtsville, NY). The O₂-hHb/PolyHb equilibrium binding curves were measured using a Hemox Analyzer (TCS Scientific Corp., New Hope, PA). The hHb/PolyHb kinetics of O₂ offloading (kₐff, O₂) were measured with an Applied Photophysics SF-17 microvolume stopped-flow spectrophotometer (Applied Photophysics Ltd., Surrey, United Kingdom) using protocols previously described by Rameez and Palmer. The MW distribution was estimated using an Acclaim SEC-1000 column (Thermo Scientific, Waltham, MA) on a Thermo Scientific Dionex Ultimate UHPLC system using previously described methods.

Dorsal chamber window model. Adult female 8- to 10-week old female BALB/c-nu/nu mice were used for the xenografted tumors according to protocols approved by the University of California San Diego Animal Care and Use Committee. Mice were instrumented with dorsal chamber windows as described previously. This experimental model is an excellent system to observe O₂ delivery as it is highly sensitive to changes in O₂ supply. Additionally, this model is especially useful when examining changes in microvascular pO₂ distributions in the unanesthetized state. Human melanomas (FME and LOX) were initiated by implanting a 200–500 μm xenograft into the fascial side of the intact skin layer of the chamber window model. FME and LOX cells were generously donated by Micro-Target Dynamic Therapy (San Diego, CA). The FME and LOX human melanoma cell lines were originally developed by the Rofstad Group at the Institute for Cancer Research (Oslo University Hospital, Norway). After implantation of the xenografts, tumors were allowed to grow for 7 days before analysis. Mice were divided into two groups based on the implanted human tumor cell lines (FME or LOX). Each of these groups was further subdivided into three cohorts: (1) an unsupplemented baseline, (2) infusion of 30:1 R-state PolyHb, and (3) infusion of 35:1 T-state PolyHb. At this point, mice underwent a 20% top load (20% of the mouse blood volume calculated by weight) infusion of a 30:1 R-state PolyHb or a 35:1 T-state PolyHb at a concentration of 100 mg/mL. PolyHb was infused via tail vein injection. After infusion, the animal was placed into a restraining tube. Once in the tube, the protruding chamber window was fixed to a microscopic stage of a BXS1W1 intravital microscope (Olympus, New Hyde Park, NY). Tissue images were...
then projected to a 4,815 charge-coupled device camera (Cohu Industries, Poway, CA). A LUMPFL-WIR×40 numerical 0.8 aperture water immersion objective (Olympus, New Hyde Park, NY) was used to carry out the measurements. Mean arterial pressure (MAP) and heart rate (HR) were recorded using a pressure transducer connected to the femoral artery catheter using an MP-150 system (BIOPAC Systems, Goleta, CA). Arterial blood was collected in heparinized capillary tubes and immediately analyzed for PaO2, PaCO2, and pH using a 248 Blood Chemistry Analyzer (Bayer, Norwood, MA). Total Hb was measured spectrophotometrically using a B-hemoglobin Hemocue (Stockholm, Sweden). Plasma Hb was measured from plasma collected after the capillary tube was centrifuged.

**Phosphorescence quenching microscopy (PQM).** Phosphorescence quenching microscopy (PQM) was used to analyze the O2 distribution in the tissue and vascular space, as described previously58. This high-resolution method allows us to resolve the pO2 of arterioles and venules within the growing tumor. To determine the pO2 in this method, we measure the decay rate of the excited palladium-mesotetra-(4-carboxyphenyl) porphyrin (Frontier Scientific Porphyrin Products, Logan, UT) bound to albumin. We then used the measured fluorescence lifetime (τp), fluorescence lifetime in the absence of O2 (τp,0), and fluorescence quenching rate constant (kq) to calculate the pO2 using the Stern–Volmer equation, as shown in Eq. (1)59.

\[
pO2 = \frac{\tau_{p,0} \tau_p}{\tau_{p,0} k_q}
\]

The probe was injected intravenously 10 min before pO2 distributions were measured to allow time for the phosphorescent probe to circulate and diffuse into the chamber window model. The exposed tissue within the chamber window was then excited with 420 nm wavelength light. To acquire τp, the 680 nm emitted phosphorescence signal was collected. Because this method is relatively independent of the probe concentration, we were also able to measure extravascular tissue pO2.

**Microvascular hemodynamics.** To observe changes in the arteriole and venule diameter, we used a video image shearing method to determine blood vessel diameter60. Center-line velocities of arterioles and venules were measured with a 102B Vista Electronics photo-diode velocity tracker (San Diego, CA) using a cross-correlation method. Volumetric flow rate (Q) through the arterioles and venules was then calculated using the radius of the vessel (rves) and average fluid velocity (vf), as described in Eq. (2). For these calculations, we assume that fluid velocity profile was parabolic in arterioles and venules.

\[
Q = \pi r_{ves}^2 v_f
\]

We are also able to calculate the oxygen extraction fraction (OEF) from the various species (dissolved oxygen, RBCs, PolyhHb) in solution by calculating the average mass flux of O2 into the tumor subtracted by the blood flow normalized average mass flux of O2 out of the tumor. The resulting O2 mass deficit is then divided by the average mass flux of O2 into the tumor as shown in Eq. (3).

\[
OEF = \frac{\sum [O_{2,in}]_n Q_{in}}{n \sum [O_{2,in}]_n Q_{in} - \sum \sum [O_{2,mem}]_m Q_{m} \sum Q_{out}}
\]

**Tumor growth model.** Similar to the chamber window model study, adult 8- to 10-week old female BALB/c-nu/nu mice were used for the xenografted tumors according to protocols approved by the University of California San Diego Animal Care and Use Committee. Approximately 4 × 10⁷ cells of the human melanoma cell lines FME and LOX were injected into the mouse flank. Mice were divided upon the tumor cell lines (FME or LOX). Each of these groups was further subdivided into three cohorts: (1) an unsupplemented baseline, (2) infusion of 30:1 R-state PolyHb, and (3) infusion 35:1 T-state PolyHb. Mice were infused with the 100 mg/mL PolyHb solutions via tail vein injection of 20% of the mouse’s blood volume once each week during the study. During tumor growth, the length of the tumor (L_tumor) and width of the tumor (W_tumor) were both measured to estimate tumor volume as shown in Eq. (4).

\[
V_{tumor} = \frac{\pi L_{tumor} W_{tumor}^2}{6}
\]

**Tumor blood flow.** Fluorescently labeled microspheres were used to estimate tumor blood flow in tumors as described previously61. In short, 15 μm diameter fluorescent microspheres (Molecular Probes, Eugene, OR) were suspended in saline. 100 μL of this solution was rapidly injected into the animal via the tail vein. Arterial reference samples were simultaneously withdrawn at a constant rate of 100 μL/min for 1 min through an inserted femoral catheter. At the end of the protocol, the mice were euthanized with a lethal dose of sodium pentobarbital. Tumor tissue was then digested in 1 M KOH solution for 24 h. Fluorescent dye was extracted with Cellosolve (Fisher Scientific, Pittsburgh, PA). The fluorescent signal was then measured using an LS 50B luminescence spectrometer (PerkinElmer Corp., Norwalk, CT). Regional blood flow proportional to the fraction of cardiac output was calculated by measuring the number of fluorescent microspheres in the tumor tissue relative to the total in the arterial reference samples.
Tumor histopathology. Harvested tumors were fixed in PBS 4% paraformaldehyde, embedded in paraffin cases, cut into sections, and stained. Vascular density was assessed by counting the number of capillary profiles within a 0.8 mm² field of view. Positive capillaries were only counted if a lumen and a brown staining endothelial cell were identified.

Statistical analysis. All results are presented as the mean ± standard deviation. Statistically significant changes were analyzed with two-way ANOVA followed by post-hoc analysis using Tukey’s multiple comparison test when appropriate. All statistics were calculated with R (v 3.6.2). Results were considered statistically significant if P < 0.05.

Ethics approval. All experimental protocols used to handle the mice were approved by the University of California San Diego Institutional Animal Care and Use Committee. The Hb used to prepare these materials was purified from expired RBCs donated from the Wexner Medical Center (Columbus, OH).

Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Received: 12 March 2020; Accepted: 17 June 2020
Published online: 09 July 2020

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The authors declare no competing interests.

Implementation of the experiment. All authors reviewed the manuscript. P.C. and A.P. reviewed and revised the manuscript. All authors contributed to the design and

Acknowledgements
We would like to thank Cynthia Walser for the surgical preparations and tumor implantation. The LOX and FME human melanoma cell lines were generously donated by Micro-Target Dynamic Therapy (San Diego, CA). This work was supported by the following: National Institute of Health Grants R56HL123015, R01HL126945, R01HL138116, and R01EB021926; National Cancer Institute Grant R01CA188652; and the Pelotonia Graduate Research Fellowship. Any opinions, findings, and conclusions expressed in this material are those of the author(s) and do not necessarily reflect those of the Pelotonia Fellowship Program.

Author contributions
D.B. wrote the main manuscript text and prepared the figures. D.B. and A.L. performed the experiments and analyzed the data. P.C. and A.P. revised and revised the manuscript. All authors contributed to the design and implementation of the experiment. All authors reviewed the manuscript.

Competing interests
The authors declare no competing interests.
