Research article

Biocompatibility of the oxygen carrier polymerized human hemoglobin towards HepG2/C3A cells

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ABSTRACT

Hemoglobin (Hb) based oxygen carriers (HBOCs) are designed to minimize the toxicity of extracellular Hb, while preserving its high oxygen-carrying capacity for oxygen delivery to cells. Polymerized human Hb (PolyHb) is a novel type of nanosized HBOC synthesized via glutaraldehyde-mediated crosslinking of free Hb, and which preserves the predominant quaternary state during the crosslinking reaction (low oxygen affinity tense (T) quaternary state PolyHb is synthesized at 0% Hb oxygen saturation, and high oxygen affinity relaxed (R) quaternary state PolyHb is synthesized at 100% Hb oxygen saturation). Major potential applications for PolyHbs, and HBOCs in general, include oxygenation of bioreactor systems containing large liver cell masses, and ex-vivo perfusion preservation of explanted liver grafts. The toxicity of these compounds toward liver cells must be evaluated before testing their use in these complex systems for oxygen delivery. Herein, we characterized the effect of PolyHbs on the hepatoma cell line HepG2/C3A, used as a model hepatocyte and as a cell line used in some investigational bioartificial liver support devices. HepG2/C3A cells were incubated in cell culture media containing PolyHbs or unmodified Hb at concentrations up to 50 mg/mL and for up to 6 days. PolyHbs were well tolerated at a dose of 10 mg/mL, with no significant decrease in cell viability; however, proliferation was inhibited as much as 10-fold after 6 days of exposure at 50 mg/mL. Secretion of albumin, and urea, as well as glucose and ammonia removal were measured in presence of 10 mg/mL of PolyHbs or unmodified Hb. In addition, methoxy- and ethoxy-resorufin deacetylase (MROD and EROD) activities, which reflect cytochrome P450 metabolism, were measured. R-state PolyHb displayed improved or intact activity in 3 out of 7 functions compared to unmodified Hb. T-state PolyHb displayed improved or intact activity in 4 out of 7 functions compared to unmodified Hb. Thus, PolyHbs, both in the R-state and T-state, are safer to use at a concentration of 10 mg/mL as compared to unmodified Hb in static culture liver-related applications.

1. Introduction

Oxygen delivery is critical to support cellular functions in various liver bioengineering applications such as bioartificial livers, *ex vivo* whole liver organ perfusion, and other tissue-engineered liver systems [1–3]. Oxygen tension is also thought to be a key modulator

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of metabolic zonation in the liver [4]. Because oxygen is sparingly soluble in water and most biological fluids, supraphysiological oxygen levels are often used to maximize oxygen delivery, thus resulting in potential oxygen toxicity via the generation of reactive oxygen species (ROS) [5,6]. Furthermore, unless high perfusion flow rates are used, the cells may be exposed to a steep and non-physiological oxygen gradient. Conversely, high perfusion flow rates cause mechanical shear damage to the cells. While whole red blood cells (RBCs) have sometimes been used as oxygen carriers in *ex vivo* applications, especially for whole liver perfusions, this approach is limited by the supply of RBCs, pump-induced hemolysis, as well as issues related to blood storage and immune compatibility [7]. Artificial oxygen carriers may overcome these limitations, as they could be more widely available and have longer *ex vivo* storage shelf lives with simpler storage requirements. Among those are perfluorocarbon (PFC) emulsions and hemoglobin (Hb)-based oxygen carriers (HBOCs). PFCs have had numerous side effects, while HBOCs have received more attention in recent years [7–9].

HBOCs have shown comparable efficacy to RBCs in normothermic machine perfusion of human donor livers [10,11]. HBOCs have also been tested as RBC substitutes in orthopedic surgeries [12–14]. Extracellular Hb is known to be toxic to human cells without the protective mechanisms of RBCs that prevent Hb dimerization and heme iron oxidation [15], and adverse events using HBOCs due to nitric oxide (NO) scavenging, hyperoxygenation, and heme-mediated toxicity have been reported in clinical studies [16]. One way to decrease toxicity is by increasing the molecular size of the HBOC thus limiting its ability to extravasate into the tissue space. This may be accomplished by chemical crosslinking multiple Hb molecules together, thus yielding a high molecular weight (MW), yet nanosized, polymerized Hb (PolyHb) [17]. Furthermore, crosslinking Hb under 0% and 100% oxygen saturation facilitates the synthesis of tense (T) and relaxed (R) quaternary state PolyHbs, thus resulting in different products with different oxygen-binding affinities, which can be mixed together to allow the release of bound oxygen over a wide range of oxygen tensions [18,19]. While the oxygen-carrying properties of glutaraldehyde cross-linked PolyHb have been thoroughly evaluated [20,21], the cytotoxicity of these materials on cells remains unknown.

In this study, we evaluated the effects of PolyHbs on the human hepatoma cell line HepG2/C3A. This cell line was used as a model human liver parenchymal cell, which performs a variety of liver-specific functions and has also been used in some experimental bioartificial liver systems [22–24]. These studies enabled us to identify the tolerable concentration of PolyHbs towards HepG2/C3A cells, thus defining the limit on the amount of PolyHb that can be used to increase the oxygen-carrying capability of culture media in static cell culture, and more broadly provide guidance on how to use PolyHbs as an oxygen carrier in liver bioreactors, liver organ perfusion systems, and with engineered liver culture systems.

2. Materials and methods

2.1. HepG2/C3A cell culture

HepG2/C3A cells were obtained from the American Type Culture Collection (ATCC) and maintained following their recommendations. All cells used in experiments were of a maximum passage number of 10 in the laboratory. Cells were seeded and cultured in complete cell culture media consisting of Eagle's Minimum Essential Media (EMEM; ATCC 30-2003) supplemented with 10% v/v fetal bovine serum (FBS; Gibco 26140079) and 1% v/v Penn/Strep (Gibco 15140122) until specific treatments were applied. Cell culture in this study was conducted at 37 °C in a Thermo Forma 3140 IR CO_2/O_2 water-jacketed incubator with 5% CO_2 and >90% humidity.

2.2. Preparation of PolyHbs

PolyHb was prepared in the R and T quaternary states as described elsewhere [20,25,26]. Briefly, expired human RBCs were purchased from the Columbus, Ohio branch of the American Red Cross, and then subjected to hypotonic lysis to release human Hb. Purified human Hb was crosslinked with glutaraldehyde while fully oxygenated or deoxygenated to yield 30:1 (molar ratio of
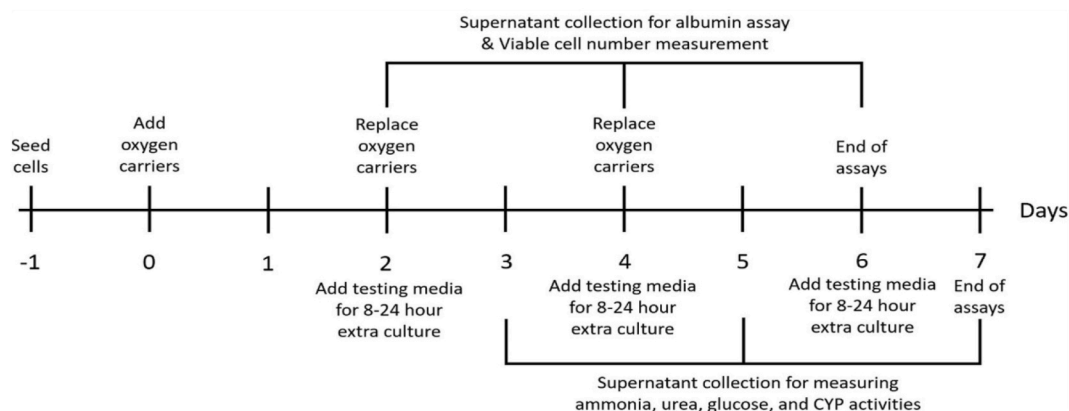

Fig. 1. General timeline for all cell function evaluation assays.

glutaraldehyde to hHb) tense (T) and 30:1 relaxed (R) quaternary state PolyHb, respectively, following further purification using multi-step tangential flow filtration over hollow fiber filter modules (Repligen Co., Waltham, MA). The oxygen carriers were prepared in modified Ringer's lactate solution. Stock solutions of PolyHb and unmodified Hb contained 100 mg/mL of oxygen carrier and were stored at $-80\,^{\circ}$C. They were thawed at $4\,^{\circ}$C overnight before use. Physical properties of PolyHbs/Hb were measured for quality control purposes. Hb concentration and methemoglobin (metHb) level of PolyHb were determined by a cyanmethemoglobin assay [27] (Supplemental Material 1).

2.3. Incubation of HepG2/C3A cells with PolyHbs/Hb

HepG2/C3A cells were seeded and allowed to attach and proliferate for 24 h before beginning treatment on day 0 (Fig. 1). Then the cell-seeding media was replaced with fresh complete cell culture media containing the oxygen carrier (T- or R-state PolyHb, unmodified Hb, or 10% (v/v) modified Ringer's lactate solution as the vehicle control) to begin exposure. Cells were directly exposed to the cell culture media containing PolyHbs, cell-free Hb or vehicle (shown in Figures as the control group). The media was then replaced with fresh complete media with PolyHb/Hb of the same type every 2 days until the end of the experiment, 6 days after the beginning of exposure. Supernatant samples were stored at $-80\,^{\circ}$C for later analysis. In some studies, at the desired time point (after 2, 4 or 6 days of oxygen carrier exposure), media was replaced with testing media without PolyHb/Hb for up to one day. This testing media did not contain PolyHb/Hb due to its interference with some of the biochemical assays.

2.4. Screening of effect of PolyHbs/Hb on HepG2/C3A cell proliferation

HepG2/C3A cells were seeded at low density, i.e. 5000/well in 96-well plates, and allowed to attach and recover for one day. Then, cells were exposed to 200 μL media supplemented with PolyHb (either R-state or T-state) or unmodified Hb at concentrations ranging from 10 to 50 mg/mL. Controls consisted of 10% v/v modified Ringer's lactate vehicle. The number of viable cells was measured after 2, 4, and 6 days of exposure to the oxygen carrier. An Alamar Blue assay (Thermofisher) was used to measure total cellular metabolic activity per well at each time point. Wells seeded with 100 to 10,000 cells/well were used to generate a standard curve of cell number vs. Alamar Blue signal. Raw data were then converted to a cell number and expressed as a fold increase in cell number relative to the initial number of seeded cells.

2.5. Supernatant collection and cell counting

In all liver-related functional assays, 1 mL supernatant for each condition was collected by pipette transfer to micro centrifuge tubes on days 2, 4, and 6, and stored at $-80\,^{\circ}$C. Wells were fed fresh culture media containing the same oxygen carriers, with the exception that at each time point, some wells were sacrificed for cell counting. Cells were briefly washed with PBS 3 times, then trypsinized, stained with 0.4% trypan blue, and viable/dead cell numbers were determined using an automated cell counter (Countess II, Thermofisher).

2.6. Effect of PolyHbs/Hb on albumin secretion

HepG2/C3A cells were seeded at 3 million/well in 12-well plates and cultured for 1 day to confluence. Cells were treated with 1 mL complete media containing 10 mg/mL PolyHb, unmodified Hb, or vehicle Ringer's lactate, with media collected and replenished every other day. Cell culture supernatants were assayed for albumin concentration in a sandwich-type enzyme immunosorbent assay (ELISA) kit (EHALB, Invitrogen) by following the manufacturer's instructions.

2.7. Effect of PolyHbs/Hb on metabolic activities

HepG2/C3A cells were first cultured the same way as described in 2.6, with exception that on days 2, 4, and 6, the media (containing PolyHb, unmodified Hb, or vehicle) was replaced with testing media of composition described below.

For urea production and ammonia removal, the 1 mL testing media consisted of 2 mM ammonium chloride (Sigma Aldrich) in phenol red-free Minimum Essential Media (MEM, Gibco 51200038) supplemented with 2% v/v FBS (Gibco, 26140079), 1% v/v Penn/Step (Gibco, 15140122), 1% v/v sodium pyruvate (Gibco, 11140050), and 1% v/v Non-essential Amino Acids (Gibco, 11360070). Cells remained in this testing media for 24 h, which was then collected to measure urea and ammonia levels. The modified Jung's method (BioAssay Systems, DIUR-100) was used to measure urea concentration, and ammonia levels were measured with an Enzychrome ammonia/ammonium assay kit (BioAssay Systems, ENH3-100).

For glucose consumption, the 1 mL testing media consisted of complete media without PolyHbs/Hb. After 8 h of incubation, the concentration of remaining glucose was measured with a glucose colorimetric detection kit (Invitrogen, EIAGLUC) and compared to that of fresh media (~1 mg/mL) to calculate the amount of glucose consumed.

2.8. Effect of PolyHbs/Hb on cytochrome P450 (CYP) activity

A 7-ethoxy-resorufin*O*-demethylation (EROD) assay was used to determine the activity of CYP1A1, and a 7-methoxy-resorufin *O*-demethylation (MROD) assay was used to determine the activity of CYP1A2 [28–30]. For this purpose, HepG2/C3A cells were seeded

at 3 million/well in 12-well plates and cultured for 1 day to confluence. Then, cells were cultured in 1 mL complete media containing 10 mg/mL PolyHb, unmodified Hb, or vehicle modified Ringer's lactate supplemented with 1 μM 3-methylcholanthrene (3-MC, Sigma Aldrich, 213942) as a CYP inducer. On days 2, 4, and 6, the media (containing PolyHbs/Hb and CYP inducer) was replaced with 1 mL testing media consisting of complete media with 10 μM 7-ethoxy-resorufin or 7-methoxy-resorufin, and 10 μM dicumarol. After 5 h of incubation, the testing media supernatants were collected and mixed with a deconjugation mixture consisting of 1 part β-glucur-onidase/arylsulfatase (Roche, BGALA-RO) diluted in 100 parts of 0.1 M fresh sodium acetate buffer (S7899, Sigma Aldrich) (pH = 4.5), followed by incubation on a shaker at 37 °C for 2 h before quenching the reaction with methanol. Fluorescence was measured (excitation 530 nm, emission 585 nm) and converted to a concentration of resorufin produced based on a standard curve. 7-ethoxy-r-esorufin (E3763), and 7-methoxy-resorufin (M1544) were purchased from Sigma Aldrich and made into 10 mM stock solutions using dimethyl sulfoxide (DMSO, Sigma Aldrich, D2438) and then stored at −30 °C. They were diluted 1000X in cell culture media before use. 10 mM dicumarol (M1390, Sigma, Aldrich) was dissolved in sterile 0.1 N NaOH solution freshly before the assay and diluted 1000X in cell culture media before use.

2.9. PolyHb and Hb stability assays

Cell culture supernatants collected after each time point were snap-frozen and stored at −80 °C until further analysis. The concentrations of the intact PolyHb, unmodified Hb, free heme, and methemoglobin (metHb) were measured by absorbance spectrum analysis and appropriate standard curves following protocols previously described in detail [31,32].

3. Results

3.1. Effect of PolyHb concentration on cellular proliferation and viability

To establish the concentration of PolyHb that is safe for HepG2/C3A cells, the latter were cultured at low density to observe the proliferative response for up to 6 days in the presence of T-state PolyHb or R-state PolyHb levels ranging from 10 to 50 mg/mL. Control groups included unmodified Hb and Ringer's lactate vehicle containing no oxygen carrier. Using an Alamar blue assay to measure cellular metabolic activity as a metric of viable cell number, the fold increase in cell number from the number of seeded cells was lower as the concentration of PolyHb or unmodified Hb, as well as the exposure time, increased (Fig. 2A and B,C). At the lowest concentration of 10 mg/mL, T-state PolyHb and unmodified Hb did not cause a significant decrease in viable cell number compared to the control vehicle group. At that concentration, R-state PolyHb caused a significant decrease in viable cell number (estimated from Alamar Blue signals) on days 2 and 6, although not on day 4. PolyHb at the concentration of 10 mg/mL in this culture format did not affect viable
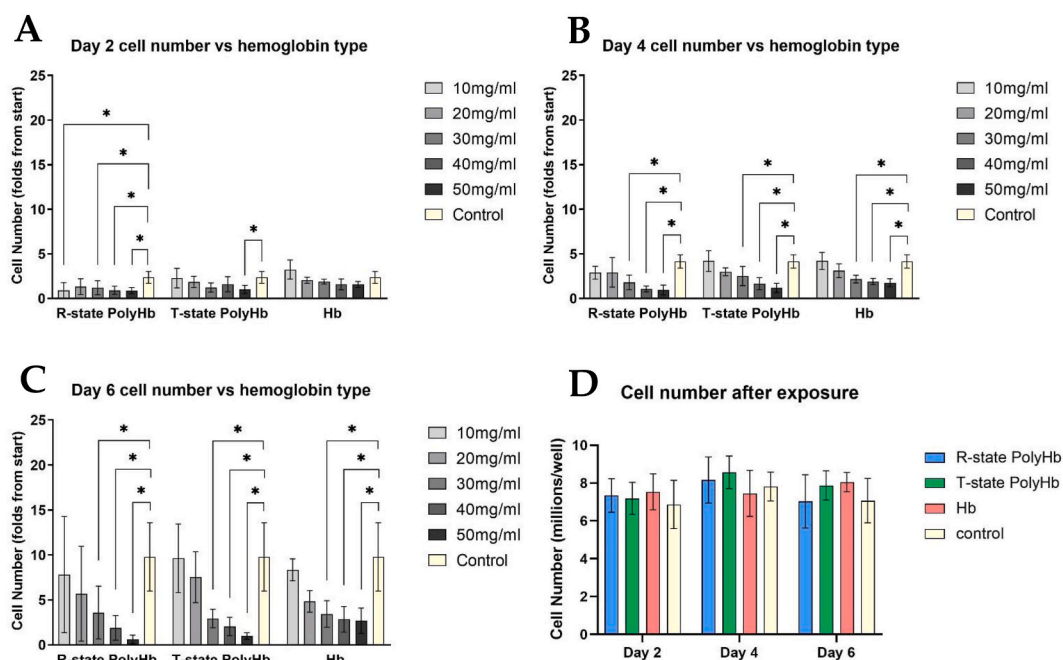

Fig. 2. Dose response of PolyHb/Hb on proliferation. **(A)**, **(B)** and **(C)** show the effect of oxygen carrier on HepG2/C3A cell proliferation for 2, 4, and 6 days, respectively. Each data point is the fold increase in cell number relative to initial. **(D)** shows viable cell numbers in each well, as measured by direct counting, when confluent HepG2/C3A cells are exposed to 10 mg/mL R-state PolyHb, T-state PolyHb or unmodified Hb for up to 6 days.

cell number (Fig. 2D).

3.2. Measurement of metabolites from HepG2/C3A cells exposed to PolyHb

Based on the prior results, the effect of PolyHbs on HepG2/C3A cell function was investigated using a PolyHb concentration of 10 mg/mL. These studies used larger format cultures and a higher seeding density in order to provide sufficient material for biochemical analysis. Albumin production decreased over time in all groups (Fig. 3A). Furthermore, compared to the vehicle control group at each time point, albumin production was decreased in groups treated by PolyHbs and unmodified Hb, with R-state PolyHb having the least effect, followed by T-state PolyHb, and unmodified Hb. This indicates that all HBOCs applied to HepG2/C3A cells had negative effects on the albumin secretion function of the cells, but the toxicity ranked as unmodified Hb > T-state PolyHb > R-state PolyHb.

3.3. Urea synthesis and ammonia removal by HepG2/C3A cells exposed to PolyHb

For these measurements, cells were exposed for 2, 4, or 6 days to PolyHb or unmodified Hb, and then switched for 24 h to testing media consisting of clear (phenol red-free) media with no PolyHb/Hb supplemented with 2 mM ammonium chloride. Both R-state and T-state PolyHb promoted urea synthesis to a greater level than cells treated with unmodified Hb and vehicle controls (Fig. 3B). Urea production with R-state PolyHb was also slightly higher than in T-state treated groups on day 6 + 1. It is noteworthy that the amount of urea produced, on the order of 1 mg/dL, corresponds to 167 μM urea produced, which would consume ~330 μM ammonia assuming the typical arginase I pathway, which is responsible for ammonia removal in hepatocytes. Ammonia concentration was however not decreased by cell culture in any of the groups when compared to blank wells with no cells (Fig. 3C). Rather, a small but not statistically significant increase was observed. Furthermore, there appeared to be no correlation between the change in ammonia concentration and the magnitude of urea production among the experimental groups.

3.4. Glucose consumption rate by HepG2/C3A cells exposed to PolyHb

Glucose consumption over an 8 h incubation period was measured in cells pre-exposed to PolyHb or unmodified Hb for 2, 4, or 6 days. Cells consumed 75–85% of the available glucose during this period (Fig. 3D). Cells exposed to R-state PolyHb consumed about 5% less glucose than the control vehicle, which was a statistically significant decrease. T-state PolyHb and unmodified Hb showed a slight, although not statistically significant, increase in glucose consumption.

3.5. CYP1A1 and CYP1A2 activities in HepG2/C3A cells exposed to PolyHb

CYP1A1 and CYP1A2 of HepG2/C3A cells were measured as described in 2.8 [28–30]. As shown in Fig. 4A, both PolyHbs and unmodified Hb significantly decreased CYP1A1 activity, as compared to controls. A similar pattern was displayed with CYP1A2
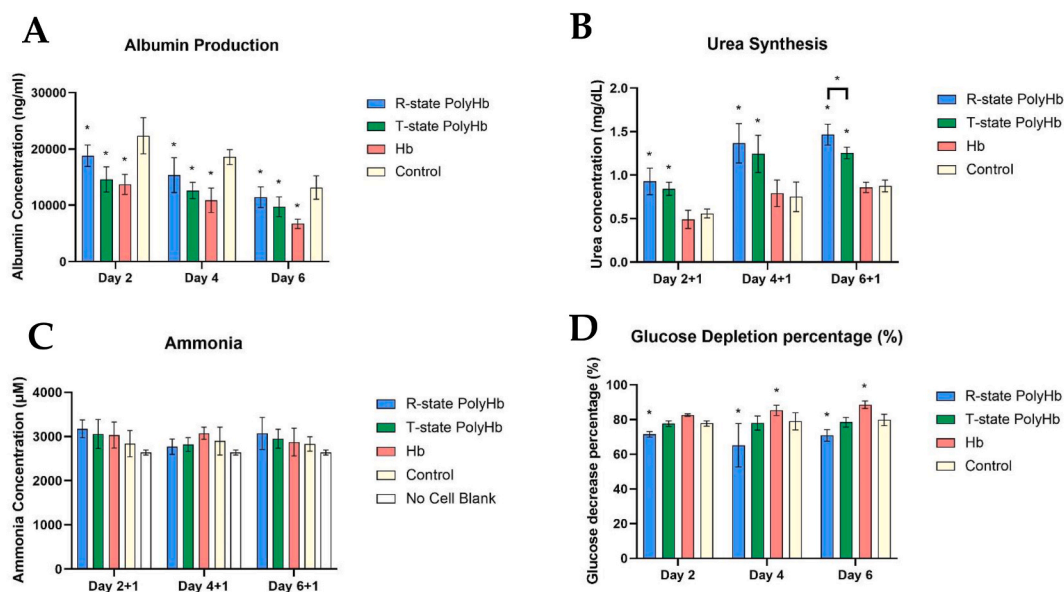

Fig. 3. Measurements of metabolites of confluent HepG2/C3A cells exposed to 10 mg/mL R-state PolyHb, T-state PolyHb or unmodified Hb for up to 6 days. **(A)** Albumin released during each 2-day incubation period. **(B)** Urea accumulation by HepG2/C3A cells over a 24 h incubation period beginning 2, 4, or 6 days after exposure. **(C)** Ammonia concentration in media from the same cultures in **(B)**. **(D)** Glucose depletion fraction over 8 h after exposure, compared to vehicle control (10% Ringer's lactate).

activity (Fig. 4B), although the decrease was less pronounced.

3.6. Stability of PolyHbs and unmodified Hb during cell culture

Media samples collected after incubation with cells for 2 days were analyzed for metHb content (Fig. 5). The initial metHb level before incubation with cells accounted for ~1.1% of the total Hb, and ~5% of the total PolyHb (PolyHbs and Hb were characterized before incubation with cells; data is available in the Supplemental Material section). That proportion increased to ~75% with un-modified Hb incubated with cells, a difference that is statistically significant. It increased to 50–60% with R-state PolyHb and T-state PolyHb, although this difference was not statistically significant vs. blanks (incubated under the same conditions without cells). More metHb was formed in the unmodified Hb group when applied in the static HepG2/C3A cell culture environment. This indicates the PolyHbs are more stable and can better preserve the oxygen-carrying capacity compared to unmodified hemoglobin under the HepG2/C3A cell culture conditions. Data collected from cell cultures that were 2 days, 4 days, and 6 days old yielded very similar results, which was expected since spent media was replaced with fresh media every time for a subsequent 2-day incubation (Fig. 1).

3.7. Statistics

Each data point in Figs. 2 and 3 (A) (D) is the average of 6 replicates ± SD. Each data point in Fig. 3 (B) (C), Figs. 4 and 5 is the average of 3 replicates ± SD. Statistics were performed by two-way ANOVA followed by post hoc multiple comparisons using Tukey's test in GraphPad Prism. *: $p < 0.05$ in the indicated comparison or vs. the control group in same treatment cohort, unless otherwise specified.

4. Discussion and conclusion

In this study, we evaluated the effects of PolyHbs and cell-free unmodified Hb on HepG2/C3A cell viability and functions (Table 1). The results herein suggest that PolyHbs have a minimal effect on the viability and proliferation of HepG2/C3A cells in static culture as long as the PolyHb concentration does not exceed 10 mg/mL. Furthermore, at 10 mg/mL, PolyHbs had mild stimulatory and inhibitory effects on specific metabolic functions of the HepG2/C3A cells, as compared to controls exposed to vehicle (10% v/v modified Ringer's lactate) (Table 1). For example, R-state PolyHb had no significant effect on albumin secretion, while it slightly decreased glucose consumption and CYP1A family activities. T-state PolyHb significantly decreased albumin secretion, did not change glucose consumption rate, and slightly decreased the CYP1A family activities. Overall, T-state PolyHb had the least impact on HepG2/C3A cells. Unmodified Hb decreased albumin secretion the most, while it slightly increased glucose consumption, and lowered the CYP1A enzyme family activities to a similar extent as R-state PolyHb did.

The concentration of 10 mg/mL used for PolyHb is approximately 15-fold less than the level of Hb in healthy human blood (~15 g/dL). The solubility of oxygen at 37 °C in plasma is ~4.3 mg/L at a partial pressure of oxygen (pO$_2$) of 100 mm Hg (typical arterial blood oxygen level), and ~280 mg/L in whole blood with ~15 g/dL Hb [33]. PolyHbs were previously found to have the same oxygen carrying capacity as native Hb, thus at a concentration of 10 mg/mL, one could expect about 20–25 mg oxygen/L media, which corresponds to about 4 times that of plain media or human plasma, and 8–10% of the oxygen carrying capacity of whole human blood [34,35].

The 4-fold higher oxygen carrying capacity of media with PolyHb allows one to use lower and possibly near-physiological perfusion flow rates in bioartificial liver (BAL) systems and liver organ systems, thus preventing high fluid shear conditions and also allowing for metabolite concentration gradients to form, which may play a role in zonation of liver function [36]. Higher concentrations of PolyHbs in the bulk fluid may also be possible to use safely if the oxygen carrier is separated from the cells using a semipermeable membrane with a low enough molecular weight cutoff to exclude PolyHbs.

Hollow fiber membranes have been widely used as semi-permeable barriers in liver bioreactors to separate the liver cells from media perfused in the hollow fiber lumen. This enables nutrient and oxygen delivery to a large number of cells with minimum interference to the cell growth environment. Many studies have focused on improving the hollow fiber materials [37]. Palakkan et al.
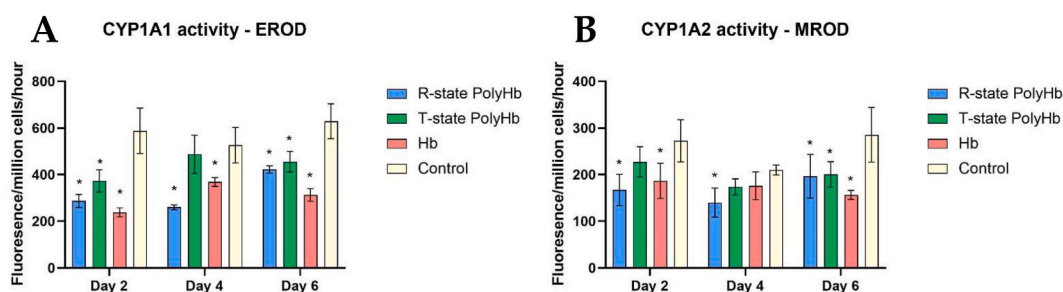

Fig. 4. **(A)** Fluorescent resorufin accumulation after incubation with 7-ethoxy-resorufin and **(B)** 7-methoxy-resorufin. All groups were compared to the vehicle control group consisting of 10% Ringer's lactate. This is a representative data set of 3 separate experiments.
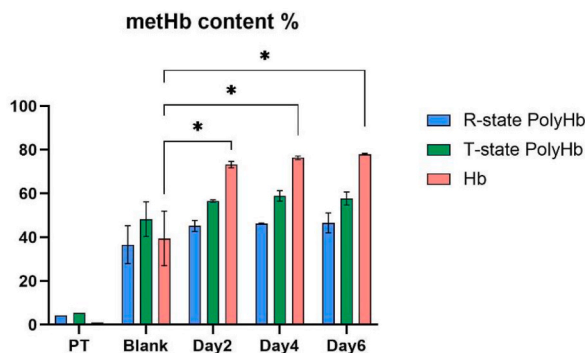
Fig. 5. Effect of incubation with HepG2/C3A cells on the percentage of metHb. Media containing R-state PolyHb, T-state PolyHb, or unmodified Hb were incubated with HepG2/C3A cells and collected on days 2, 4 and 6 for analysis of metHb content. Blank controls were incubated without cells for 2 days. PT group indicates the initial metHb content (%) in the PolyHb and Hb solutions prior to incubation with cells.

Table 1

Summary of the effects of R-state PolyHb, T-state PolyHb, and unmodified Hb at 10 mg/mL on HepG2/C3A functions (-: no change), compared to a vehicle control (the control group: cell culture media containing 10% (v/v) Ringers' lactate solution).

	R-state PolyHb	T-state PolyHb	Unmodified Hb
Proliferation	↓	–	–
Albumin secretion	–	↓	↓
Urea synthesis	↑	↑	–
Ammonia removal	–	–	–
Glucose consumption	↓	–	↑
CYP1A1 activity	↓	↓	↓
CYP1A2 activity	↓	↓	↓
metHb formation	↑	↑	↓

have shown that hollow fiber liver bioreactors can support large numbers of HepG2 cells if perfused at a sufficiently high rate with cell culture media [38]. A slower flow rate may however be possible when using PolyHbs, due to the increased oxygen carrying capacity. This would lead to greater changes in other metabolite levels across the length of the bioartificial liver device, which may resemble more closely the metabolite gradients that exist *in vivo* across the liver acinus. Hepatocytes exposed to an *in vivo*-like metabolic micro-environment may better emulate their in-vivo functions thus improving the performance of BAL systems [39]. For similar reasons, the use of PolyHbs may be advantageous for normothermic preservation of perfused liver explants [40,41].

It is well known that low molecular weight glutaraldehyde PolyHbs, such as Oxyglobin, are toxic [42–44]. Oxyglobin induces systemic hypertension and oxidative tissue injury. To counter these side-effects, recent data showed that increasing the molecular weight of PolyHb reduced vasoconstriction and systemic hypertension [17], and decreased kidney injury [45] by preventing PolyHb extravasation into the tissue space. This would also help minimize the cytotoxicity in BAL applications mentioned previously, due to the size-based separation of PolyHbs and cells by a semipermeable membrane.

Additionally, PolyHbs would likely undergo degradation pathways similar to that for extracellular Hb *in vivo* [46], and thus are not expected to exhibit unexpected long-term toxicity problems that have been reported with emulsified perfluorocarbons (PFCs), a fully synthetic oxygen carrier that has long organ retention times [47,48]. Furthermore, reports indicate that PFCs strongly inhibit cytochrome P450 mediated detoxification functions in liver cells [49]. Cytochrome P450 (CYP) activity is a critical function of phase I drug metabolism, which only occurs in the liver. Recapitulating and/or maintaining hepatocyte drug metabolic functions is one of the main goals of bioartificial livers and *ex vivo* liver perfusion. Although we observed partial loss in cytochrome P450 activity with PolyHbs (Fig. 4), it could be compensated by increasing cell mass in a BAL if necessary. When comparing the R-state PolyHb group to the Hb group, they had similar CYP activity, and the difference was not significant. The T-state PolyHb group generally exhibited higher CYP function, although it was still lower than the baseline level in the control group. This study eliminated the effect of oxygen tension by culturing the cells in an open-air setting and focused on the biological effects from PolyHbs only. At this point, the reason why T-state PolyHb performs better than R-state towards CYP is unclear. In addition to CYP1A1 and 1A2, we also tried to measure CYP2B6 and CYP3A4 activity, which are also representative CYP activities in hepatocytes that are preserved in immortalized HepG2/C3A cells, albeit at a level that is 2 orders of magnitude lower than in primary hepatocytes [50]. The signals were undetectable using resorufin substrate-based assays (as shown in the Supplemental Material 2 section).

Immortalized HepG2/C3A cells have been previously used to seed certain cell-based BAL systems due to their availability and human origin [24]. It is noteworthy that, in this study, PolyHbs promoted urea synthesis without a concomitant effect on ammonia removal, which is consistent with the high activity of the arginase II pathway in these cells, while ammonia conversion to urea in the liver is mainly carried out by the arginase I pathway [51]. Note that urea synthesis was promoted by PolyHbs (Fig. 3B); however, the

mechanistic connection between PolyHb and arginase II is unclear at this time. Since arginase II is a mitochondrial enzyme (as opposed to arginase I, which is cytosolic), a potential route for further investigation may involve increased oxygen delivery to the cells in the presence of the oxygen carrier [52,53].

MetHb is the form of Hb where Fe^{2+} has converted to the Fe^{3+} state due to auto-oxidation of the heme group. PolyHb and un-modified Hb in the metHb form lack oxygen-carrying capability [54]. Although conversion to metHb during cell culture was observed in all groups, PolyHbs exhibited less metHb formation, suggesting that glutaraldehyde-mediated crosslinking of Hbs in PolyHb renders Hb less vulnerable to oxidation. It is noteworthy that the fraction of metHb in cell-free Hb incubated samples reached ~40%, while the typical metHb fraction of freshly thawed PolyHb is ~5% after purification [55]; thus, auto-oxidation is nevertheless quite significant and the addition of antioxidants and/or reducing agents (e.g. ascorbic acid) may be considered to reduce oxidation of PolyHb [42].

In conclusion, we show that HepG2/C3A cells tolerate well direct exposure to PolyHbs at a concentration of 10 mg/mL in static cell culture. PolyHbs generally exhibited less cytotoxicity as compared to unmodified Hb. The results showed that for eight different measured parameters, both PolyHbs showed significant improvement in 3 of them (PolyHb stability, albumin secretion, and urea synthesis) compared to unmodified Hb. PolyHbs had no significant effect on ammonia removal and glucose consumption. T-state PolyHb had similar to improved effect on cell proliferation compared to unmodified Hb, and showed a less negative effect, compared to unmodified Hb, on CYP activities. R-state PolyHb had a negative effect on proliferation compared to unmodified Hb.

Although there was some minor loss of function in PolyHb-treated cells, those changes could be compensated by small increases in cell mass in liver tissue engineering applications. Furthermore, in applications where PolyHbs are not in direct contact with the cells, such as when the oxygen carrier is physically separated from the cells by a nanoporous membrane, higher concentrations of PolyHbs may be used, as long as the cell-containing compartment remains at levels below 10 mg/mL. PolyHb stability and toxicity may be further decreased using biocompatible anti-oxidants such as ascorbic acid. PolyHbs may be useful in BAL systems and *ex vivo* liver perfusion systems [2,56].

We determined that 10 mg/mL is a safe concentration towards HepG2/C3A cells in terms of biocompatibility. The benefit of O_2 supply from PolyHbs remains unknown in this study, but this finding provides guidance for future studies that will explore the impact of PolyHbs' oxygen-carrying capability in 3-dimensional culture systems containing large masses of liver cells, for attempted *ex vivo* liver perfusion, or for liver-on-chip devices for drug screening.

Author contribution statement

Nuozhou Chen: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Donald A. Belcher; Chintan Savla: Performed the experiments.

Andre F. Palmer: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Francois Berthiaume: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Funding statement

Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of competing interest

All authors declare that they have no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e15878.

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