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TITLE: Simultaneous Monitoring of Vascular Oxygenation and Tissue Oxygen Tension of Breast Tumors Under Hyperbaric Oxygen Exposure

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**Objective/Hypothesis:** By monitoring global and local vascular oxygenation and tissue oxygen tension in breast tumors under HBO exposure with several different gas interventions, we wish to prove the following two hypotheses: that 1) the combination of HBO and hyperoxic intervention can largely improve breast tumor oxygenation, and that 2) tumor oxygenation remains elevated for a period of time even after HBO exposure, which may provide a unique treatment window to enhance radio-sensitivity.

**Specific Aims:**

**Aim 1:** to determine the absolute values of oxygenated hemoglobin concentration, [HbO₂], and hemoglobin oxygen saturation, SO₂, in solid breast tumors from the NIRS measurements.

**Aim 2:** to investigate vascular oxygenation and tissue oxygen tension of breast tumors under continuous normobaric and hyperbaric oxygen exposures with several gas interventions, using both a single-channel NIRS system and 3-channel FOXY pO₂ system simultaneously.

**Aim 3:** to investigate global and local dynamics of tumor vascular [HbO₂] and tissue pO₂ of breast tumors immediately after HBO exposure using both three-channel NIRS and 19F MR EPI imaging simultaneously.

**SUBJECT TERMS**

Technology Development, Near infrared spectroscopy, Hyperbaric Oxygen Treatment, Tumor Therapy Planning and Prognosis, Tumor Physiology Monitoring.

**ABSTRACT**

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2004-2008 FINAL PROGRESS REPORT

This report presents the specific aims and accomplishments of our breast cancer research project during the years of funding sponsored by the US Department of the Army. It covers our activities from May 1, 2004 to April 30, 2008.

1. Introduction

The overall goal of this research project is to apply the multiple monitoring techniques, i.e. Near infrared spectroscopy (NIRS), FOXY oxygen sensor and $^{19}$F MR EP imaging of Hexafluorobenzene (HFB), to prove the following hypotheses: combination of hyperbaric oxygen (HBO) intervention can significantly improve breast tumor oxygenation, and that tumor oxygenation remains elevated for a substantial period of time even after HBO exposure, which may be a novel approach to enhance radiosensitivity or chemotherapy. If our hypotheses are proven to be true, this study can lead to an optimal intervention plan to improve tumor oxygenation and to determine an optimal time interval after HBO decompression for radiotherapy. Such a novel approach will largely enhance the efficiency of non-surgical therapies for breast tumor treatment and provide a novel prognostic tool for clinical practice. This study will also provide a better understanding of tumor vasculature and tissue oxygen dynamics and spatial heterogeneity under HBO exposure.

The project has three specific aims:

Aim 1: to determine the absolute changes of oxygenated hemoglobin concentration, $[\text{HbO}_2]$, and hemoglobin oxygen saturation, $\text{SO}_2$, in solid breast tumors from the NIRS measurements.

Aim 2: to investigate vascular oxygenation and tissue oxygen tension of breast tumors under continuous normobaric and hyperbaric oxygen exposures with several gas interventions, using both a single-channel NIRS system and 3-channel FOXY pO$_2$ system simultaneously.

Aim 3: to investigate (1) whether HBO could enhance the therapeutic efficiency of malignancy when used as a chemotherapeutic adjuvant of an anticancer agent (doxorubicin, DOX) in mammary carcinomas of rat model, and (2) the feasibility of NIRS to monitor tumor hemodynamic changes resulting from the therapeutic effect of DOX on vasculature.

This report will summarize the work we performed for the three specific aims and corresponding achievements, as given below.

2. Body of the Report

The PI and her mentor, Ms. Mengna Xia and Dr. Liu, made significant efforts from 2004-2008 to accomplish the proposed aims, resulting in multiple published journal and conference papers. This report is organized in such a way that we will combine our published papers and Dr. Xia’s dissertation to give detailed descriptions of the research studies, while the papers and chapters in her dissertation are organized as Appendixes 1 to 4 for referral, as listed below:


Appendix 2: Yueqing Gu, Wei R. Chen, Mengna Xia, Sang W. Jeong, and Hanli Liu, “Effect of Photothermal therapy on breast tumor vascular contents: non-


2.1 Report for Aim 1

**Aim 1:** to determine the absolute changes of oxygenated hemoglobin concentration, \([\text{HbO}_2]\), and hemoglobin oxygen saturation, \(\text{SO}_2\), in solid breast tumors from the NIRS measurements.

For Aim 1, we have implemented quantification algorithms, examined their corresponding errors, and conducted animal measurements to obtain absolute changes of oxygenated hemoglobin concentration, \([\text{HbO}_2]\), and hemoglobin oxygen saturation, \(\text{SO}_2\), in solid breast tumors based on the NIRS measurements. The detailed descriptions can be found in Appendixes 1 to 3.

2.2 Report for Aim 2

**Aim 2:** to investigate vascular oxygenation and tissue oxygen tension of breast tumors under continuous normobaric and hyperbaric oxygen exposures with several gas interventions, using both a single-channel NIRS system and 3-channel FOXY \(\text{pO}_2\) system simultaneously.

For Aim 2, we have reported our experimental results in Appendixes 1 and 4 for investigation of vascular and tissue oxygenation of rat breast tumors under continuous normobaric (Appendix 1) and hyperbaric (Appendix 4) oxygen exposures with oxygen interventions, using single-change NIRS and multi-channel \(\text{pO}_2\) sensors simultaneously. The details can be found in those two appendixes.

2.3 Report for Aim 3

**Aim 3:** to investigate (1) whether HBO could enhance the therapeutic efficiency of malignancy when used as a chemotherapeutic adjuvant of an anticancer agent (doxorubicin, DOX) in mammary carcinomas of rat model, and (2) the feasibility of NIRS to monitor tumor hemodynamic changes resulting from the therapeutic effect of DOX on vasculature.

For Aim 2, the PI, Dr. Mengna Xia, has reported the detailed results in her dissertation. Here we report the major findings briefly, as follows:

2.3.1 Introduction

Doxorubicin (DOX) is one of the most widely used broad-spectrum anticancer agents [1]. However, its clinical utility is limited, because this agent produces a chronic and dose-related
cardiomyopathy as its principal side effect. Therefore, it is desirable to achieve better chemotherapeutic effect with lower dosage of the agent. It is well accepted that hypoxic tumor is resistant to radiotherapy and some chemotherapy agent [2,3,4,5]. To overcome hypoxia, a variety of approaches have focused on improve oxygen delivery via oxygen-enriched gases or blood substitutes [6,7,8,9,10]. Hyperbaric oxygen was believed to improve tissue oxygenation greater than normobaric oxygen because it increased oxygen tension and oxygen delivery to tissue independent of hemoglobin[11]. HBO, as a chemotherapy adjuvant in tumor treatment rather than stand alone treatment, is believed to increases cellular uptake of some chemotherapy agents and the susceptibility of cells to these agents. It has been demonstrated that HBO can increase the susceptibility of malignant cells to destruction with taxol [12], doxorubicin [13, 14] and 5-FU [15,16].

The influence of tumor oxygenation on treatment outcome has stimulated various techniques to monitor or estimate tumor oxygenation. These include microelectrodes, optical reflectance, electron paramagnetic resonance (EPR), magnetic resonance imaging (MRI) and nuclear medicine approaches, as reviewed previously [17]. As each approach has their own strength, some are highly invasive. Since its introduction in 1970s [18], Near Infrared Spectroscopy has been increasingly applied to study tissue oxygenation status non-invasively. Near infrared light can easily penetrate biological tissue, and allow for detection of specific light-absorbing chromophores in human in vivo, such as oxygenated and deoxygenated hemoglobin, water and lipid [19]. it has been used extensively for quantitative measurements of cerebral oxygenation [20] and blood oxygenation in muscles in vivo [21], and more recently, tumor vascular oxygenation with respect to interventions [22]. NIRS currently lack of spatial resolution, and thus, the utility of global measurement require validation, given the well-documented heterogeneity of tumor. In this regard, Xia et al [22] compared the spatially averaged measurement of relative tumor oxygen saturation ($S_O^2$) using NIRS with the local $pO_2$ measured by MRI. The sensitivity and specificity analysis suggests that NIRS may identify clinically relevant hypoxia, even when its spatial extent is below the resolution limit of the NIRS technique. Kim et al demonstrated that NIRS may be used as an effective tool to monitor tumor hemodynamic change induced by some vascular disrupting agent [23,24].

These studies were designed to investigate 1) whether HBO could enhance the therapeutic efficiency of malignancy when used as a chemotherapeutic adjuvant of doxorubicin in mammary carcinomas of rat model, and 2) the feasibility of NIRS to monitor tumor hemodynamic changes resulting from the therapeutic effect of DOX on vasculature.

### 2.3.2 Materials and Methods

#### 2.3.2a Animal and Tumor models

Healthy female Fischer 344 rats aged 5 to 6 months were obtained from Harlan Sprague-Dawley (Indianapolis, IN). They were housed for at least a week to acclimatize and for monitoring of health before inclusion in the study. Mammary carcinomas 13762NF were implanted in the dorsum of female Fischer 344 rats weighing ~200g. Tumor volume was estimated by the formula of $6/\pi * (L \times W \times H)$. Tumor diameter was measured in orthogonal axes ($L$, $W$, $H$). Tumor size and body weight were monitored every other day after therapeutic interventions. All animal protocols were approved by Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center and University of Texas at Arlington.
2.3.2b Drug preparation and dose

Doxorubicin Hydrochloride was purchased from Sigma Aldrich, Inc. It was made into a solution by dissolving with saline. A single dose of DOX (2mg/kg body weight) was administrated by tail vein. The dose of doxorubicin given was the usual chemotherapeutic dose, which would not cause cardio-toxicity [13].

2.3.2c Experimental procedure

Following tumor establishment (~1 cm diameter), rats were randomly assigned to one of three groups according to different therapeutic strategies: a) DOX (n=5), b) HBO + DOX (n=5). C) control group (n=2) with saline injection. Rats were anesthetized with the mixture of ketamine hydrochloride (0.15 ml; 100mg/ml; Aveco, Fort Dodge, IA) and xylazine via i.p. After tumor hair was shaved to allow better optical contact for NIR light transmission, the rat was placed on its side in the hyperbaric chamber. Probes of SSDRS were fixed securely on the tumor of rat, and then SSDRS monitored tumor vascular oxygenation while the rat was subjected to therapeutic interventions.

DOX group was given 0.4 mg/ml doxorubicin solution intravenously after the respiratory intervention of air - O₂ - air, and then were exposed to air – O₂ - air after DOX injection. HBO + DOX group was exposed to gas intervention in a sequence of air-O₂ - HBO (30 min) prior to intravenously administration of 0.4 mg/ml doxorubicin solution, and then exposed to air - O₂ – air. Control group was subjected to the respiratory intervention of air -O₂ - air, saline solution intravenous injection and then were exposed to air – O₂ – air. To control the timing of DOX injection, an IV butterfly catheter was inserted into rat tail vein and fixed securely with tape before the rat was put into the chamber. The syringe filled with DOX solution was connected to the catheter with heparin only before DOX injection, in order to avoid the possible precipitation caused by the incompatibility of DOX and heparin [25].

2.3.2d Spectrometer for monitoring the disturbance of DOX on the absorption of tissue phantom

Because the reddish color of doxorubicin, it is reasonable to consider the bolus injection of doxorubicin may change tissue absorption. A UV/VIS spectrometer Lambda 20 (PerkinElmer Inc., Waltham, MA) was used to detect the absorption change resulting from the addition of DOX into tissue phantom, in order to verify the acute effect of DOX bolus injection on tissue absorption. A tissue phantom is composed of 100 l sheep blood mixed with phosphate buffer solution with total volume of 3.5 ml and total hemoglobin concentration of 7.1 g/L, which is in the range of total hemoglobin concentration in tissue [26]. The Hb concentration of tissue phantom was measured by Co-oximeter (Instrumentation Lab, Ramsey, MN). Since animal’s total blood volume is 10% of its body weight, total blood volume = 0.2 Kg x 10% = 20 ml, with the known body weight of ~ 0.2 Kg of rats. 0.17 ml DOX solution with the concentration of 0.4 mg/ml was added into the tissue phantom. Accordingly, the volume ratio of DOX to tissue phantom is proportional to the ratio of 1 ml 0.4 mg/ml DOX to 20 ml total blood volume in rats. According to the Lambert Beer Law, the absorption in 750nm and 830nm are

\[
A^{750}_0 = \varepsilon^{750}_{Hb} [Hb] + \varepsilon^{750}_{HbO_2} [HbO_2] \\
A^{750}_1 = \varepsilon^{750}_{Hb} [Hb] + \varepsilon^{750}_{HbO_2} [HbO_2] + \varepsilon^{750}_{DOX} [DOX] \\
A^{830}_0 = \varepsilon^{830}_{Hb} [Hb] + \varepsilon^{830}_{HbO_2} [HbO_2]
\]

(2.1) 
(2.2) 
(2.3)
\[ A^{830}(1) = \varepsilon_{Hb}^{830}[Hb] + \varepsilon_{HbO_2}^{830}[HbO_2] + \varepsilon_{DOX}^{830}[DOX] \]  

(2.4)

where \( A^{750}(0) \) and \( A^{750}(1) \) represent the absorption at 750 nm in tissue phantom without DOX and with DOX, respectively. \( A^{830}(0) \) and \( A^{830}(1) \) represent the absorption at 830 nm in tissue phantom without DOX and with DOX, respectively.

2.3.2e Steady-state diffuse reflectance spectroscopy (SSDRS) for measuring changes in tumor vascular oxygenation (\( \Delta[HbO_2] \))

A broadband diffuse reflectance spectrometer was used to acquire reflectance spectra from tumor tissue. Briefly, continuous wave (CW) light from a 20 W tungsten-halogen light source (HL-2000HP, Ocean Optics, FL) is coupled into a 2.6-mm core diameter fiber optic bundle, the distal end of which is placed in physical contact with the surface of the tumor. After being scattered in the tumor tissue, the transmitted light is collected by a 1-mm core diameter detection fiber, the end of which is coupled to a hand-held spectrometer (USB2000, Ocean Optics, FL). The broadband light diffuse spectrometer provides reflectance spectra from 400 to 900 nm.

According to the modified Beer-Lambert law, changes of oxy- and deoxy-hemoglobin concentration, \( \Delta[HbO_2] \) and \( \Delta[Hb] \), can be derived from the measured amplitudes at two wavelengths (750nm and 830nm), by using extinction coefficients of oxy- and deoxy-Hb published by Cope [27], as given in Equations. (2.5) and (2.6):

\[
\Delta[HbO_2] = \frac{-1.532 \log(\frac{A_h}{A_t})^{750} + 1.753 \log(\frac{A_h}{A_t})^{830}}{DPF \cdot d} 
\]  

(2.5)

\[
\Delta[Hb] = \frac{1.758 \log(\frac{A_h}{A_t})^{750} - 0.92 \log(\frac{A_h}{A_t})^{830}}{DPF \cdot d} 
\]  

(2.6)

where \( A_b \) is the baseline amplitude, \( A_t \) is the transient amplitude during the intervention, and \( d \) is the direct source-detector separation. \( DPF \) (differential path-length factor) is a tissue-dependent parameter and defined as the ratio between the optical path length and the physical separation between the source and detector.

2.3.3 Results

2.3.3a Disturbance of DOX on the absorption of tissue phantom

There is absorption difference between tissue phantom with and without DOX, as shown in Figure 2.1. However, the differences are relatively small. Indeed, the relative changes of absorption in wavelength of 750nm and 830nm are \( \frac{A^{750}(1) - A^{750}(0)}{A^{750}(0)} = 4.85\% \) and
\[ \frac{A_{830}(1) - A_{830}(0)}{A_{830}(0)} = 4.80\% \], respectively. In order to investigate the disturbance of DOX on calculating oxygen saturation, absorption spectrum profiles between 700 and 900 nm were normalized by absorption values at the wavelength of 700 nm. The normalized spectra cover the wavelengths we utilized for calculating hemoglobin concentration. As shown in figure 2.2, profiles of normalized absorption spectrum in NIR range appeared to be overlaid.

Figure 2.1 Absorption spectra of tissue phantoms with (yellow curve) or without 170 ul DOX (blue curve). The unit for absorption is arbitrary unit.

Figure 2.2 Normalized absorption spectra of tissue phantoms with (yellow curve) or without 170 ul DOX (blue curve) in the wavelength range of 700 ~ 900 nm.

2.3.3b Changes in Tumor volume and body weight during chemotherapy

Tumor volume and body weight were monitored before and after DOX treatment to examine the tumor response. Changes in tumor volume and body weight were normalized by the values at day 0 (before DOX administration).
Tumors in the control group grow significantly faster than tumors with treatment in the other groups \( (p<0.05) \), as shown in Figure 2.3a. Tumors with combined treatment of HBO and DOX grow significantly slower than those with DOX treatment except for the first two days after treatment \( (p<0.05) \). Basically, there is no significant difference for tumor volume in HBO + DOX and DOX groups on the 2nd day after treatment. However, significant differences are observed starting from 5th day after treatment between the DOX treated and control group \( (p<0.05) \). Regarding body weight loss, figure 2.3b indicated that rats in DOX group and DOX + HBO group had significant and continuous body weight loss after day 0. Rats in control group gained weight at day 2, and started to lose weight after day 4, and then kept constant body weight thereafter.

2.3.3c Vascular hemodynamic changes of rats in DOX group
Values of $\Delta [\text{HbO}_2]$ of rats in DOX group showed increases with values in a range of $0.07 \sim 0.12$ (mM/DPF), when the gas switched from air to oxygen before DOX injection. When the gas was switched from air to oxygen after DOX administration, the maximal amplitude of $\Delta [\text{HbO}_2]$ increased with values in a range of $0.02 \sim 0.04$ (mM/DPF), which is significantly less than the increase achieved with $\text{O}_2$ inhalation before DOX administration, as shown in figure 2.4. We also noticed the signal changes during DOX i.v. injection.

Figure 2.4  Time profile of $\Delta [\text{HbO}_2]$ of tumors (1~4) in DOX group when the rat was under gas intervention. The unit for $\Delta [\text{HbO}_2]$ is mM/DPF. $\Delta [\text{HbO}_2]$ of the 5th rat was discarded because of misplaced gas mask during the experiment due to the movement of the rat.
2.3.3d Vascular hemodynamic changes of rats in DOX + HBO group

Figure 2.5 Typical time profile of $\Delta[HbO_2]$ of tumors in DOX + HBO group (n=5) when the rat was under gas intervention.
It shows a stable baseline in $\Delta[HbO_2]$ when rats were inhaling air. $\Delta[HbO_2]$ increase immediately in the first few minutes and more gradually afterwards, as shown in figure 2.5. When the rats were exposed to hyperbaric oxygen, $\Delta[HbO_2]$ has a further increase until reaching a stabilized value. DOX i.v. injection immediately after HBO caused some fluctuation of signal, but stabilized when the injection is finished. $\Delta[HbO_2]$ has a stable baseline but with values greater than air inhalation before HBO when the rats were breathing air after HBO. Similar to DOX group, the maximal amplitude of $\Delta[HbO_2]$ achieved with $O_2$ inhalation prior to the DOX administration is significantly greater than that achieved with $O_2$ inhalation after DOX administration.

2.3.3e Vascular hemodynamic changes of rats in control group

$\Delta[HbO_2]$ have stable baseline values when rats were breathing air, as shown in figure 2.6. $\Delta[HbO_2]$ increased when the gas was switching to oxygen, and decreased when the gas was switched back to air, as expected. Different from $\Delta[HbO_2]$ in DOX treated group, the change of amplitude in $\Delta[HbO_2]$ when the gas switched from air to oxygen after saline injection is similar to the change due to gas intervention before injection in both rats.

2.3.4 Discussion and Conclusion

In our study, we randomized breast tumors into three groups: group with DOX injection, group with DOX injection following HBO exposure at 2 atm, control group with saline injection. The result showed that tumors in HBO + DOX group grow significantly slower than those with DOX
alone. HBO enhanced chemotherapeutic response of mammary carcinoma NF13762 to DOX in vivo reflected by slowing down the tumor growth after treatment.

Resistance to chemotherapy is common in hypoxic tumors. HBO may help overcome chemotherapy resistance by improving both tumor perfusion and cellular sensitivity. Improving tumor oxygenation and vascularization may increase drug delivery. This has been shown experimentally and in nude mice with human epithelial ovarian cancer treated with cisplatin [28]. Reactive oxygen species (ROS), or free radicals are by-product of aerobic respiration and cellular metabolism and induced by oxidative stress during hypoxia (oxygen deficiency), reperfusion or hyperoxia (excess oxygen). ROS, at low levels, assist tumor growth but become toxic at high levels. This can be explained by the “threshold effect” whereby ROS reach a level beyond which the antioxidant capacity is inundated, resulting in irreversible damage and apoptosis [29,30]. One of the mechanisms of action of doxorubicin is production of ROS. By increasing ROS level, HBO push ROS levels past the threshold level, and thus enhanced the ROS-localized effects of doxorubicin [31,32]. Another mechanism of HBO is to push cell to enter a proliferate stage, thus sensitizing them to radiotherapy and some chemotherapy by improving oxygenation. It has been showed that HBO enhanced the chemotherapeutic effects of doxorubicin in an experimental model of pulmonary sarcoma [13]. HBO stimulated proliferation of an MCA-2 metastatic lung tumor cell line and induced cells to enter the replicating cycle compared to cells left at ambient pressure [13]. Other studies found that HBO increased the ratio of prostate cancer cells in vitro accumulating in G2/M phases from the G0 arrest phase [33]. Generally speaking, HBO therapy in combination with chemotherapy may be justified by the following: 1) improved oxygenation improves drug delivery to hypoxic regions in the tumor; 2) increasing intratumoral ROS levels beyond the threshold may induce tumor destruction; 3) improved oxygenation may also cause cell to enter a proliferate stage, thus sensitizing them to radiotherapy and some chemotherapy; 4) HBO may remove hypoxia stimulus that drives angiogenesis.

Even though the DOX dosage is reported to cause minimal cardiotoxicity, the result showed that changes in amplitudes of Δ[HbO2] before DOX administration were much greater than the change after treatment in rats of DOX group and DOX + HBO group. It is likely that the amplitude difference results from the known cardiotoxicity reaction, the major side effect of DOX. It has been suggested that cardiac dysfunction induced by DOX resulted from the imbalances of the circulatory system such as decreases in blood pressure or the direct effects on vascular wall [34]. During the course of i.v. injection of DOX, the vasculature was exposed to high levels of DOX, and in vitro studies have suggested that DOX acutely induces vascular smooth muscle to release Ca2+ from its intracellular storage site and causes direct vasoconstrictor [35] and vasodilator effects [36]. Furthermore, the combination of doxorubicin and HBO would also be expected to enhance the agent’s cardiotoxicity because of the toxicity to cardiomyocytes of HBO. Therefore, the clinical addition of HBO to doxorubicin may not change the risk-benefit ratio of the agent. NIRS, in turn, may provide a novel approach to monitor the cardiotoxicity of treatment, which may leads to an optimized therapeutic plan to minimize the side effect of treatment. We also noticed the signal fluctuation in Δ[HbO2] during DOX injection in DOX group. DOX solution is orange-red, so it is likely that DOX bolus injection would cause the absorption change of tumor tissue in NIR range. We measured and compared the spectra of tissue phantoms before and after adding DOX, to examine the disturbance of DOX injection on the signal of Δ[HbO2]. There were absorption differences when comparing both absorption spectra (Figure 2.1). However, the normalized spectra appeared to be overlaid (Figure 2.2). Therefore, it implied that DOX would affect total hemoglobin concentration, rather than oxygen saturation.
3. Key Research Accomplishments and Reportable Outcomes

3.1 Ph.D. Dissertation:
Title: TISSUE AND VASCULAR OXYGENATION DYNAMICS DETERMINED BY OPTICAL APPROACHES AND MRI

3.2 Publications in peer-reviewed journals:

3.3 Proceeding papers and presentations:
(1) Mengna Xia, Benjamin Levine, Ralph Mason, Hanli Liu, “Simultaneous monitoring of tumor vascular oxygenation and tissue oxygen tension under hyperbaric oxygen exposure”, in Biomedical Topical Meetings on CD-ROM (The Optical Society of America, Washington, DC, 2006).

4. Conclusions
In this 4-year study, we have learned that HBO enhances the therapeutic action of doxorubicin in our tumor model, probably by multiple physiological mechanisms. The present study reveals that DOX may be used in conjunction with HBO to obtain the same effect as higher doxorubicin doses. Meanwhile, NIRS may work as an attractive approach to monitor the treatment.

Overall, our study over the last few years has demonstrated that 1) NIRS and MRI and FOXY™ oxygen sensor are complimentary approaches to monitor tumor oxygenation. 2) Tumor tissue oxygenation achieved by hyperbaric oxygenation persists over 10-20 minutes even after terminating hyperbaric oxygenation intervention. 3) Several correlations existed for both modalities (HbO₂ and pO₂) under sequences of hyperoxic gas intervention with hyperbaric oxygen exposure. Correlation of tumor vascular oxygenation and tumor tissue pO₂ determined simultaneously by those techniques could give us a better understanding on the pathophysiology of tumor and response to therapeutic interventions. 4) HBO enhanced the therapeutic action of doxorubicin in our tumor model, probably by multiple physiological mechanisms. DOX could be used in conjunction with HBO to achieve the same effect as higher doxorubicin doses. NIRS may work as an attractive approach to monitor radiation-toxicity of the treatment.
5. References


Tumour oxygen dynamics measured simultaneously by near-infrared spectroscopy and $^{19}$F magnetic resonance imaging in rats*

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Abstract

Simultaneous near-infrared spectroscopy (NIRS) and magnetic resonance imaging (MRI) were used to investigate the correlation between tumour vascular oxygenation and tissue oxygen tension dynamics in rat breast 13762NF tumours with respect to hyperoxic gas breathing. NIRS directly detected global variations in the oxygenated haemoglobin concentration ($\Delta[HbO_2]$) within tumours and oxygen tension ($pO_2$) maps were achieved using $^{19}$F MRI of the reporter molecule hexafluorobenzene. Multiple correlations were examined between rates and magnitudes of vascular ($\Delta[HbO_2]$) and tissue ($pO_2$) responses. Significant correlations were found between response to oxygen and carbogen breathing using either modality. Comparison of results for the two methods showed a correlation between the vascular perfusion rate ratio and the mean $pO_2$ values ($R^2 > 0.7$). The initial rates of increase of $\Delta[HbO_2]$ and the slope of dynamic $pO_2$ response, $d(pO_2)/dt$, of well-oxygenated voxels in response to hyperoxic challenge were also correlated. These results demonstrate the feasibility of simultaneous measurements using NIRS and MRI. As expected, the rate of $pO_2$ response to oxygen is primarily dependent upon the well perfused rather than poorly perfused vasculature.

1. Introduction

Tumour oxygenation has been widely recognized as a pivotal factor in the efficacy of radiotherapy (Hall 1994), photodynamic therapy (Chapman et al 1991) and some

* Presented in part at the 12th annual meeting of the International Society of Magnetic Resonance in Medicine, Kyoto, 2004.
chemotherapies (Brown 1999), and patient stratification with respect to tumour oxygenation status could be clinically important (Hockel et al 1996, Fyles et al 1998, Welch et al 2003). It has been hoped that modulation of tumour oxygenation could be applied to enhance therapeutic efficacy. An attractive intervention is breathing hyperoxic gas, and, indeed, several clinical trials have examined the efficacy of normobaric or hyperbaric oxygen, to improve therapeutic outcome, but often with marginal success (Overgaard and Horsman 1996). It has been suggested that outcome might have been improved, if responsive tumours could have been identified a priori. Accordingly, accurate evaluation of tumour oxygenation in response to interventions at various stages of growth should provide a better understanding of tumour response to therapy, potentially allowing therapy to be tailored to individual characteristics.

Given the importance of tumour oxygenation, many techniques have been developed based on microelectrodes, optical reflectance, electron paramagnetic resonance (EPR), magnetic resonance imaging (MRI) and nuclear medicine approaches, as reviewed previously (Mason et al 2002, Zhao et al 2004). While each approach has unique strengths, some are highly invasive. Near-infrared spectroscopy (NIRS) has been developed in recent years as a promising non-invasive technique to quantify the concentration of tissue chromophores, such as oxygenated and deoxygenated haemoglobin, water and lipid (Sevick et al 1991, Liu et al 2000). Due to the deep penetration depth and biochemical specificity of NIRS, it has been widely applied for quantitative measurements of cerebral oxygenation (Delpy and Cope 1997, Yodh and Boas 2003) and blood oxygenation in muscles in vivo (Homma et al 1996). Recently, NIRS has been also used to monitor tumour vascular oxygenation with respect to interventions (Hull et al 1999, Liu et al 2000, Gu et al 2003). With notable exceptions (Chance 1997, Dehghani et al 2004, Shah et al 2004, Pogue et al 2003), NIRS currently lacks spatial resolution, and, thus, the utility of global measurements requires validation, given the well-documented heterogeneity of tumour oxygenation. In this regard, Conover et al (2000) compared the spatially averaged measurement of tumour oxygen saturation (SO2) using NIRS with the local SO2 in individual blood vessels measured by cryospectrophotometry. The sensitivity and specificity analysis suggests that NIRS may identify clinically relevant hypoxia, even when its spatial extent is below the resolution limit of the NIRS technique. We have previously investigated correlates between pO2 assessed by electrodes (Mason et al 2003, Kim et al 2003b) or fibre-optic probes and NIRS (Gu et al 2003). On occasion, there was a good correlation between global vascular oxygenation and local pO2 at individual locations, but often, disparate behaviour was observed. Sequential MRI and NIRS suggested a better relationship based on average pO2 from multiple locations (Kim et al 2003b). We have now implemented simultaneous NIRS and 19F MRI to examine the relationships further. Since NIRS is entirely non-invasive it would provide an attractive surrogate for monitoring tumour oxygenation, and, hence, we seek correlations with absolute pO2 measurements observed simultaneously by MRI.

2. Materials and methods

Investigations were approved by the Institutional Animal Care and Use Committee.

2.1. Animal preparation and experimental set-up

Mammary adenocarcinomas 13762NF (cells originally obtained from the Division of Cancer Therapeutics, NCI) were implanted in skin pedicles (Hahn et al 1993) on the foreback of ten adult female Fisher 344 rats (~150 g). When the tumours reached ~1 cm in diameter, the rats were anaesthetized with ketamine hydrochloride i.p. (100 mg kg−1 body weight, Aveco, Fort
Dodge, IA) and were maintained under general gaseous anaesthesia (air and 1% isoflurane; Baxter International Inc, Deerfield, IL). Tumour hair was trimmed to give good optical contact for NIR light transmission. Hexafluorobenzene (HFB, 50 µl, 99.9%, Lancaster Co, Pelham, NH) was administered along two or three tracks in central and peripheral regions of the tumours in a single plane (transverse to the rat’s tumour, and in the region of NIR photon pathway) using a Hamilton syringe with a 32 G needle. The needle was inserted manually to penetrate across the tumour and was withdrawn ∼1 mm to reduce pressure and 3–4 µl of HFB were deposited. The needle was then repeatedly withdrawn 1–2 mm and further HFB deposited at each point, as described in detail previously (Zhao et al 2004).

The tumour was placed inside a size-matched Helmholtz coil, specially designed for the simultaneous MRI-NIRS study. The tumour was inserted between the two loops of the Helmholtz coil and two NIRS probes were introduced through the ends of the coil along the coil axis (figure 1). The probes were positioned to be in the same plane as the HFB injection. The rats were placed in the magnet on their side, and body temperature was maintained using a warm water blanket. A total of ten rats were used in the study: seven rats were subjected to respiratory challenge in the sequence of air–oxygen–air–carbogen–air, one rat breathed air–carbogen–air–oxygen–air, one rat breathed air–carbogen–air, and one breathed air–oxygen–air.

2.2. NIRS for measuring Δ[HbO₂]

A homodyne frequency-domain system (NIM, Philadelphia, PA) was used to monitor the global change of deoxy- and oxyhaemoglobin concentration (Δ[HbO₂]) in the tumour, as described previously (Yang et al 1997), though with minor modifications to ensure MR compatibility (figure 1). Briefly, the laser light, which was emitted from two laser diodes (at 758 nm and 785 nm), was amplitude-modulated at 140 MHz and time gated on and off sequentially. The two time-shared laser beams illuminated the tumour surface alternately through a light-delivery fibre bundle (source fibre) with a 7 m length and a 3 mm bundle diameter. The long fibre bundle ensured the separation of the NIRS hardware from the magnet. After being absorbed and scattered in the tumour tissue, the transmitted light was collected on the opposite side of the tumour by another fibre bundle (detector fibre) of same length and diameter as that of source
fibre. The collected optical signal was detected and amplified by a photomultiplier tube (PMT). The probe sheaths at the fibre tips were made of nylon for MR compatibility. An In-phase and quadrature (I/Q) demodulator chip was used to demodulate the amplitude-modulated signal from the PMT.

In principle, since the I/Q system could give both phase and amplitude values, it should be possible to calculate absolute HbO₂, Hb and SO₂ (Yang et al 1997). However, given the tumour’s small size and large spatial heterogeneity, we are currently unable to achieve reliable absolute quantification using the conventional algorithm (Fishkin and Gratton 1993) based on the diffusion approximation. Instead, based on modified Beer–Lambert’s law, we simply use the amplitude of the light transmitted through the tumour to calculate concentration changes in HbO₂, Hb and Hb of the tumour caused by respiratory intervention. Namely, changes of oxy- and deoxyhaemoglobin concentration, \( \Delta[HbO_2] \) and \( \Delta[Hb] \), respectively, can be derived from the measured amplitudes at the two wavelengths (758 nm and 785 nm) (Kim et al 2003b)

\[
\Delta[HbO_2] = -10.63 \log \left( \frac{A_b}{A_t} \right)_{758} + 14.97 \log \left( \frac{A_b}{A_t} \right)_{785} \text{DPF \cdot d}
\]

\[
\Delta[Hb] = 8.95 \log \left( \frac{A_b}{A_t} \right)_{758} - 6.73 \log \left( \frac{A_b}{A_t} \right)_{785} \text{DPF \cdot d}
\]

where \( A_b \) is the baseline amplitude, \( A_t \) is the transient amplitude during measurement and \( d \) is the direct source–detector separation. DPF (differential path-length factor) is a tissue-dependent parameter and is defined as the ratio between the optical path length and the physical separation between the source and detector. The units for \( \Delta[HbO_2] \), \( \Delta[Hb] \) and \( \Delta[Hb]_{\text{total}} \) are mM. DPF is a variable depending on tissue type and wavelengths and it is currently difficult to quantify DPF for tumours. Since this study focuses on dynamic changes of \( [HbO_2] \), \( \Delta[HbO_2] \) values may be scaled by a factor of DPF (in units of mM/DPF), to obtain characteristic features of tumour oxygen dynamics (Liu et al 2000).

2.3. Mathematical model for blood oxygenation dynamics of tumours

Based on our previous experimental study, we derived a simple mathematical model (Liu et al 2000) to examine tumour vascular dynamics during oxygen intervention, by analogy to the method used to quantify regional cerebral blood flow (rCBF) with diffusible radiotracers, originally developed by Kety (1951). Accordingly, changes of oxyhaemoglobin concentration in tumour vasculature induced by hyperoxic gas intervention, \( \Delta[HbO_2] \), can be expressed as

\[
\Delta[HbO_2(t)]_{\text{vasculature}} = \gamma H_0 \left[ 1 - \exp \left( -\frac{ft}{\gamma} \right) \right] = A \left[ 1 - \exp \left( -\frac{t}{\tau} \right) \right]
\]

where \( \Delta[HbO_2] \) corresponds to the changes in oxyhaemoglobin concentration from tumour vasculature measured by NIRS, \( H_0 \) is the arterial oxygenation input for \( \Delta[HbO_2]_{\text{artery}} \) after time 0, \( f \) is the blood perfusion rate and \( \gamma \) is the vascular coefficient of the tumour, defined as the ratio of \( [HbO_2] \) changes in the vascular bed to that in the veins. Specifically, \( \gamma = \frac{\Delta[HbO_2]_{\text{vasculature}}}{\Delta[HbO_2]_{\text{vein}}} \). \( \tau \) is the time constant (= \( \gamma / f \)) and \( A = \gamma H_0 \).

Given that solid tumours often develop hypoxic regions, which are poorly perfused (Jiang et al 2004, Mason et al 1994, Mazurchuk et al 1999, Song et al 2002), we hypothesized that the observed, bi-phasic feature of \( \Delta[HbO_2] \) came from two different perfusion regions (well- and poorly perfused regions). Therefore, it is reasonable to define two different blood
Tumour oxygen dynamics in vasculature and tissue

perfusion rates \((f_1, f_2)\) with two different vascular coefficients \((\gamma_1, \gamma_2)\) in the mathematical model. Consequently, equation (3) has been modified to a summation of two exponential expressions, representing two perfusion regions, as

\[
\Delta [\text{HbO}_2(t)]_{\text{vasculature}} = \gamma_1 H_0 \left[ 1 - \exp \left( -\frac{f_1 t}{\gamma_1} \right) \right] + \gamma_2 H_0 \left[ 1 - \exp \left( -\frac{f_2 t}{\gamma_2} \right) \right] = A_1 \left[ 1 - \exp \left( -\frac{t}{\tau_1} \right) \right] + A_2 \left[ 1 - \exp \left( -\frac{t}{\tau_2} \right) \right] \tag{4}
\]

where \(f_1\) and \(\gamma_1\) are the blood perfusion rate and the vasculature coefficient, respectively, in the well-perfused region, and \(f_2\) and \(\gamma_2\) represent the same respective meanings in the poorly perfused region. Also, it follows that \(A_1 = \gamma_1 H_0\), \(A_2 = \gamma_2 H_0\), \(\tau_1 = \gamma_1 / f_1\) and \(\tau_2 = \gamma_2 / f_2\). Since \(A_1, A_2, \tau_1\) and \(\tau_2\) can be determined by curve-fitting equation (4) to the dynamic NIRS measurements, we can obtain the ratios for two vascular coefficients and two blood perfusion rates, as follows (Liu et al. 2000):

\[
\frac{\gamma_1}{\gamma_2} = \frac{A_1}{A_2}, \quad \frac{f_1}{f_2} = \frac{A_1/\tau_1}{A_2/\tau_2}. \tag{5}
\]

These two ratios provide insight into tumour vascular structures and blood perfusion rates. Specifically, \(\gamma_1/\gamma_2\) may be associated with the vascular volume fraction between the two regions, and \(f_1/f_2\) reflects the perfusion ratio between the two regions. For example, if \(f_1/f_2\) is substantially greater than 1, the two compartments have significantly different perfusion rates.

This mathematical model is consistent with experimental animal studies (Liu et al. 2000, Gu et al. 2003, Kim et al. 2003b). Moreover, it is supported by computer simulations (Kim and Liu 2005) and a phantom study (Kim and Liu 2004) each of which shows that the bi-phasic or bi-exponential feature can be present if both a slow and a fast perfusion of flow co-exist within the interrogated volume.

2.4. FREDOM for measuring \(pO_2\)

MRI was performed using a Varian Inova 4.7 T horizontal bore system equipped with actively shielded gradients. Shimming was performed on the tumour tissue water signal to reduce the line-width to less than 100 Hz. \(^1\)H MRI (200.1 MHz) T1-weighted reference images were acquired with TR/TE of 150/10 ms and \(40 \times 40\) mm\(^2\) field of view. Following \(^1\)H MRI, corresponding \(^19\)F MR images (188.3 MHz) with a matrix size of \(32 \times 32\) (1.25 mm per pixel) were obtained to show the distribution of HFB in the tumour. A single 10 mm thick slice was chosen to include all the injected HFB, as the plane of injection was not always perfectly aligned with the imaging plane. The actual volume interrogated is defined by the distribution of the reporter molecule in the third dimension rather than the imaging gradients (Zhao et al. 2004). The FREDOM (Fluorocarbon Relaxometry by Echo-planar imaging for Dynamic Oxygen Mapping) approach was used to measure \(pO_2\), as described in detail previously (Hunjan et al. 2001). The spin lattice relaxation rate \((R1 = 1/T1)\) of HFB is highly sensitive to changes in \(pO_2\), but not sensitive to temperature variations: a deviation of 1 °C in temperature will introduce a deviation of only 0.13 Torr in the \(pO_2\) estimate, when \(pO_2\) is about 5 Torr (Zhao et al. 2004). T1 maps were computed on a voxel-by-voxel basis using nonlinear least-squares data fitting by the Gauss–Newton method. We applied a threshold to the raw T1 data in order to remove random noise, i.e., voxels with T1 error > 3.6 s or T1 error/T1 > 50% were disregarded. Maps of \(pO_2\) values with 1.25 mm pixel size were obtained from the T1 maps using the equation, \(pO_2 = (1/T1 - 0.0835)/0.001876\) (Hunjan et al. 2001).
Each pO2 map was acquired in 64 min. Three baseline pO2 data sets were acquired over 24 min for all tumours, while the rats breathed air, after which the rats were repeatedly exposed to oxygen or carbogen (95% O2 and 5% CO2) interventions. Five pO2 maps were obtained during each subsequent gas switch period. Typically, for a five gas-intervention sequence (e.g., air–oxygen–air–carbogen–air), a total of 23 pO2 maps were obtained over a period of 3 h. Due to thresholding, some voxels did not appear in all pO2 maps and these were discarded. For temporal analysis, voxels were selected as only those which provided consistently reliable data (i.e., satisfied all the thresholding criteria specified above) for all 23 measurements over the time course with a range of 5–44 acceptable voxels for the ten tumours. The slope of dynamic pO2 changes (rate) was defined as d(pO2)/dt and d(pO2′)/dt in response to increasing or decreasing inhaled FO2 (Fraction of O2), respectively.

2.5. Statistical analysis

Linear regression analysis was used to calculate the correlation between the NIRS-derived tumour haemodynamic parameters (i.e., Δ[HbO2], A1/τ1, A2/τ2, f1/f2) and the FREDOM-determined tumour parameters (i.e., pO2, d(pO2)/dt, d(pO2′)/dt). Data are presented as mean ± standard deviation (SD) and paired Student-t tests compared the effects of oxygen and carbogen on Δ[HbO2] and pO2.

3. Results

3.1. Dynamic response of Δ[HbO2] measured by NIRS

Figure 2 shows a group-averaged, temporal profile of Δ[HbO2] from seven 13762NF rat breast tumours with air–oxygen–air–carbogen–air intervention, displaying apparent biphasic responses to both interventions. Both single (equation (3)) and double-exponential (equation (4)) curve fitting were tested for the carbogen intervention in one representative tumour (1.6 cm3), as in figure 3(A). The maximal Δ[HbO2] achieved with oxygen challenge was compared with that of carbogen, and revealed no significant difference between oxygen and carbogen interventions (p > 0.3); indeed, there was a strong correlation between the
maximal $\Delta [\text{HbO}_2]$ values with these two interventions ($R^2 > 0.75$, figure 3(B)), consistent with our previous observations (Gu et al. 2003). No correlation was found between the perfusion rate ratio ($f_1/f_2$) and tumour size ($R^2 = 0.16$). Vascular oxygen dynamics in response to interventions are provided for individual tumours in table 2.

### 3.2. $pO_2$ measurements by FREDOM

Overlay of $^{19}$F and $^1$H MR images demonstrated that HFB was distributed in both central and peripheral regions of tumours (not shown). Individual $pO_2$ values taken from each voxel ranged from hypoxic ($<1$ Torr) to 35 Torr under baseline conditions (figure 4). Mean baseline $pO_2$ values, which are averaged over all voxels in a given tumour in the first three $pO_2$ maps, ranged from hypoxic ($<5$ Torr) to 27 Torr with a hypoxic fraction (HF$_5$; fractional voxels that are less than 5 Torr) ranging from 0 to 100% (mean 36%) and summarized in table 1. A strong correlation was found between mean baseline $pO_2$ and HF$_5$ ($R^2 > 0.85$, figure 5). Administration of oxygen or carbogen produced significant increases in tumour $pO_2$, as shown in the $pO_2$ maps, graphs and table. The tumour $pO_2$ values were averaged over the entire slice and responses to respiratory challenge for the group of seven tumours, measured simultaneously by FREDOM and NIRS, are shown in figure 2. Baseline measurements (breathing air) were generally stable, and altering the inhaled gas to oxygen or carbogen
Table 1. Oxygen tension (pO₂) in ten rat mammary 13762NF adenocarcinomas. pO₂ in baseline is the average value of all voxels in the first three maps, while pO₂ for oxygen or carbogen is the average value of all voxels in the final three pO₂ maps during exposure to the hyperoxic gas. Hypoxic fraction (HF) is the percentage of voxels with pO₂ values less than 5 Torr (HF₅) or 10 Torr (HF₁₀) and HF₅ and HF₁₀ are the average values of final three maps in baseline, oxygen or carbogen respectively. The mean pO₂ increased significantly with both oxygen and carbogen and the value was significantly higher with carbogen. HF₅ was significantly reduced with oxygen or carbogen.

<table>
<thead>
<tr>
<th>No</th>
<th>Tumour Volume (cm³)</th>
<th>Voxelsᵃ</th>
<th>Baseline (air)</th>
<th>Oxygen challenge</th>
<th>Carbogen challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pO₂ (Torr) Mean ± SD</td>
<td>HF₅ (%)</td>
<td>HF₁₀ (%)</td>
<td>pO₂ (Torr) Mean ± SD</td>
<td>HF₅ (%)</td>
</tr>
<tr>
<td>1</td>
<td>1.6</td>
<td>26</td>
<td>12 ± 3</td>
<td>24 ± 2</td>
<td>49 ± 15</td>
</tr>
<tr>
<td>2</td>
<td>0.3</td>
<td>10</td>
<td>12 ± 2</td>
<td>6 ± 2</td>
<td>15 ± 2</td>
</tr>
<tr>
<td>3</td>
<td>0.5</td>
<td>5</td>
<td>4 ± 4</td>
<td>60 ± 20</td>
<td>80 ± 0</td>
</tr>
<tr>
<td>4</td>
<td>1.3</td>
<td>14</td>
<td>&lt;1 Torr</td>
<td>100 ± 0</td>
<td>100 ± 0</td>
</tr>
<tr>
<td>5</td>
<td>0.9</td>
<td>24</td>
<td>24 ± 4</td>
<td>0 ± 0</td>
<td>8 ± 4</td>
</tr>
<tr>
<td>6</td>
<td>1.2</td>
<td>8</td>
<td>4 ± 1</td>
<td>58 ± 7</td>
<td>63 ± 0</td>
</tr>
<tr>
<td>7</td>
<td>0.8</td>
<td>24</td>
<td>27 ± 2</td>
<td>0 ± 2</td>
<td>3 ± 2</td>
</tr>
<tr>
<td>8</td>
<td>0.6</td>
<td>8</td>
<td>9 ± 6</td>
<td>50 ± 22</td>
<td>63 ± 22</td>
</tr>
<tr>
<td>9</td>
<td>0.7</td>
<td>44</td>
<td>25 ± 1</td>
<td>10 ± 5</td>
<td>26 ± 3</td>
</tr>
<tr>
<td>10</td>
<td>0.7</td>
<td>8</td>
<td>7 ± 6</td>
<td>54 ± 19</td>
<td>75 ± 22</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>0.9 ± 0.4</td>
<td>17 ± 12</td>
<td>12 ± 10</td>
<td>36 ± 33</td>
<td>52 ± 31</td>
</tr>
</tbody>
</table>

ᵃ Number of voxels that provide acceptable data for all 23 pO₂ maps.
ᵇ p < 0.01.
ᶜ p < 0.005.
ᵈ p < 0.05.
induced rapid and significant changes in both pO2 and Δ[HbO2] (p < 0.001). Upon return to air (baseline), Δ[HbO2] dropped quickly and significantly within 16 min, and then more slowly, for the next 24 min, whereas the pO2 decrease was more gradual. Altering the inhaled gas to carbogen also produced a rapid increase in both pO2 and Δ[HbO2]. Upon return to air breathing from carbogen, both Δ[HbO2] and pO2 showed a similar trend to that following oxygen. As expected, all ten tumours showed a significant increase in pO2 (average over entire slice), and decrease in hypoxic fraction (HF) in response to oxygen or carbogen inhalation. The magnitude of response to either hyperoxic gas was correlated (R^2 > 0.79), as was the maximum volume-averaged pO2 achieved with either gas (R^2 > 0.83). The rate of increase with oxygen challenge, d(pO2)/dt, was significantly faster than the return to baseline, d(pO2)/dt, for oxygen intervention (p < 0.02), but no difference was observed for carbogen (p > 0.1, figure 6). The mean pO2 values of individual tumours averaged over the final three pO2 maps during exposure to carbogen breathing were significantly higher than oxygen (p < 0.01), and the tumour hypoxic fraction was generally eliminated during carbogen breathing (n = 7 of 9 tumours, table 1).

3.3. The relationship between pO2 and Δ[HbO2] with respect to hyperoxic gas

Taken as a group of ten tumours, there was no apparent relationship between the magnitude of the change in tumour vascular oxygenation (Δ[HbO2]) and change in pO2 (R^2 < 0.1). However, if tumours were divided into two sub-populations, then two separate correlations
were found each with similar slope (figure 7(A)). There was also a correlation ($R^2 > 0.7$) between the perfusion rate ratio, $f_1/f_2$, derived from fitting the $\Delta[HbO_2]$ curve and the mean $pO_2$ values achieved with hyperoxic gas intervention (figure 7(B)). Assessment of $f_1/f_2$ is
Figure 8. \( \frac{dpO_2}{dt} \) versus \( A_1/\tau_1 \) (determined from \( \Delta[HbO_2] \) for tumours with biphasic response to interventions) showing a positive correlation \( (R^2 > 0.5) \). \( pO_2 \) values are the mean value of all well-oxygenated voxels (i.e., the maximum \( pO_2 > 10 \) Torr under oxygen or carbogen intervention) appearing in the five \( pO_2 \) maps during oxygen (●) or carbogen (□) intervention.

Table 2. Summary of vascular oxygen dynamics for the experimental tumours. Two amplitudes \( (A_1, A_2) \) and two time constants \( (\tau_1, \tau_2) \) were determined by curve-fitting the dynamic NIRS measurements using a double-exponential expression. Nine out of ten tumours were observed to have double-exponential features with either oxygen or carbogen intervention. \( A_1 \) is significantly smaller than \( A_2 \) with oxygen \( (p < 0.01) \), but no significant differences in carbogen \( (p > 0.19) \).

| No | Tumour | Double exponential fitting | | |
|----|--------|-----------------------------|---|---|---|
|    |        | \( A_1 \) (AU) | \( A_2 \) (AU) | \( \tau_1 \) (min) | \( \tau_2 \) (min) | \( R \) |
| 1\textsuperscript{a} | Oxygen | 0.11 ± 0.002 | 0.12 ± 0.002 | 0.49 ± 0.02 | 6.6 ± 0.2 | 0.94 |
|    | Carbogen | 0.15 ± 0.001 | 0.13 ± 0.002 | 0.58 ± 0.01 | 23.2 ± 1.1 | 0.94 |
| 2\textsuperscript{a} | Oxygen | 0.03 ± 0.002 | 0.11 ± 0.005 | 0.46 ± 0.18 | 25.6 ± 2.9 | 0.91 |
|    | Carbogen | – | – | – | – | |
| 3\textsuperscript{a} | Oxygen | 0.15 ± 0.001 | 0.38 ± 0.003 | 0.31 ± 0.02 | 20.1 ± 0.39 | 0.98 |
|    | Carbogen | 0.23 ± 0.005 | 0.2 ± 0.004 | 1.2 ± 0.04 | 10.8 ± 0.45 | 0.95 |
| 4\textsuperscript{a} | Oxygen | – | – | – | – | |
|    | Carbogen | 0.16 ± 0.002 | 2.3 ± 0.27 | 0.29 ± 0.02 | 155.8 ± 21 | 0.97 |
| 5\textsuperscript{a} | Oxygen | 0.12 ± 0.002 | 0.23 ± 0.002 | 0.55 ± 0.03 | 11.6 ± 0.27 | 0.94 |
|    | Carbogen | – | – | – | – | |
| 6\textsuperscript{a} | Oxygen | 0.06 ± 0.001 | 0.26 ± 0.005 | 0.13 ± 0.01 | 31.2 ± 1.1 | 0.96 |
|    | Carbogen | 0.03 ± 0.001 | 0.08 ± 0.001 | 0.04 ± 0.02 | 13.9 ± 0.6 | 0.78 |
| 7\textsuperscript{a} | Oxygen | – | – | – | – | |
|    | Carbogen | – | – | – | – | |
| 8\textsuperscript{b} | Oxygen | 0.11 ± 0.002 | 0.2 ± 0.001 | 0.24 ± 0.02 | 7.1 ± 0.1 | 0.96 |
|    | Carbogen | 0.2 ± 0.002 | 0.12 ± 0.001 | 0.49 ± 0.01 | 9.1 ± 0.2 | 0.95 |
| 9\textsuperscript{c} | Carbogen | 0.01 ± 0.0004 | 0.02 ± 0.0004 | 0.14 ± 0.02 | 3.9 ± 0.12 | 0.64 |
| 10\textsuperscript{d} | Oxygen | 0.05 ± 0.002 | 0.03 ± 0.002 | 0.46 ± 0.05 | 5.6 ± 0.56 | 0.68 |
| Mean ± SD | – | 0.11 ± 0.0068 | 0.32 ± 0.59 | 0.42 ± 0.3 | 24.9 ± 40.2 | – |

\textsuperscript{a} Air \( \rightarrow \) O\(_2\) \( \rightarrow \) air \( \rightarrow \) carbogen \( \rightarrow \) air.
\textsuperscript{b} Air \( \rightarrow \) carbogen \( \rightarrow \) air \( \rightarrow \) O\(_2\) \( \rightarrow \) air.
\textsuperscript{c} Air \( \rightarrow \) carbogen \( \rightarrow \) air.
\textsuperscript{d} Air \( \rightarrow \) oxygen \( \rightarrow \) air.
predicated on biphasic behaviour with respect to interventions, which was observed in most cases (13 of 16 measurements). There was also a positive correlation between $A_1/\tau_1$ (the fast component of biphasic $\Delta[HbO_2]$) and the $d[pO_2]/dt$ of well-oxygenated voxels (i.e., those with $pO_2$ values > 10 Torr under oxygen or carbogen intervention) ($R^2 > 0.5$, figure 8). However, no correlation was found between $d[pO_2]/dt$ and $A_2/\tau_2$ (the slow component).

4. Discussion

Integration of diverse imaging techniques can be technically challenging, since each modality has specific technical constraints and requirements. Here, the NIR system was modified to use longer optical fibres and any metal components were eliminated from the fibre tips. Due to the spatial restrictions within the bore of the magnet a Helmholtz coil was built specifically providing access to both the tumour and fibres. The fibres required sufficient flexibility to allow them to be bent through requisite angles within the confines of the magnet bore. To date there have been few reports of simultaneous data acquisition by NIR and MRI, e.g., studies of phantoms (Pogue et al. 2003), human brain (Toronov et al. 2001, Chen et al. 2003) and breast (Ntziachristos et al. 2000, Gulsen et al. 2002, Ntziachristos et al. 2002, Gu et al. 2004).

In the present study, global average $\Delta[HbO_2]$ was measured by NIRS, and $pO_2$ maps were obtained simultaneously by $^{19}$F MRI. We used transmission mode NIRS in order to interrogate deep tumour tissue. While NIRS is a global measurement, the region sampled by it is predominantly a banana-shaped region joining the locations of the source and detector (Arridge 1995, Arridge and Schweiger 1995), and we recently showed that typically 15 to 30% of the vascular volumes of rat tumours are interrogated by NIR using this configuration (Gu et al. 2005). We positioned the NIRS probes (source and detector) to be in the same plane as the HFB injection to ensure maximum overlap between the regions sampled by the two techniques. Utilizing our previously developed mathematical model (Liu et al. 2000), multiple haemodynamic parameters were derived for $\Delta[HbO_2]$ ($A_1/\tau_1$, $A_2/\tau_2$ and $f_1/f_2$) to be compared with $pO_2$. Our results demonstrate that oxygenation parameters measured from both techniques show significant and consistent elevation in tumour oxygenation during the hyperoxic gas interventions. As reported previously, the magnitude of the vascular response was similar with both hyperoxic gases (Gu et al. 2003). As expected, $\Delta[HbO_2]$ increased much faster than $pO_2$ in all ten tumours, indicating that change in tumour vascular oxygenation precedes tumour tissue oxygenation. This observation is consistent with our previous studies in this tumour type measured simultaneously by NIRS and fibre-optic probes (Gu et al. 2003), as well in the Dunning prostate R3327-AT1 tumours measured sequentially by NIRS and $^{19}$F MRI (Kim et al. 2003b).

We have previously demonstrated the application of FREDOM to monitor tumour oxygen dynamics in diverse rat prostate tumours (Zhao et al. 2001, 2002), human tumour xenografts (Mason et al. 2002) and a few breast tumours (Song et al. 2002). Here, the mean baseline was $pO_2 = 12 \pm 10$ Torr for the ten tumours, which is lower than reported previously (Song et al. 2002), but entirely consistent with the newer anaesthetic protocol (air or 21% oxygen, as opposed to 33% $O_2$ previously). There was a strong correlation between baseline $pO_2$ and hypoxic fraction (figure 5), as we have previously found using Dunning prostate R3327-HI tumours (Zhao et al. 2001). The $pO_2$ achieved with carbogen in this study was significantly higher than with oxygen and carbogen appeared to be more effective at eliminating the hypoxic fraction (table 1). However, carbogen was generally applied second in our experimental protocols, and it is highly likely that the initial oxygen primed the tumour. Indeed, oxyhaemoglobin generally returned to baseline during the air breathing episode between hyperoxic gases, it is clear that $pO_2$ remained elevated (figure 2). Both $\Delta pO_2$ and the
Tumour oxygen dynamics in vasculature and tissue

maximum pO\textsubscript{2} achieved with either gas were closely correlated. Similar behaviour was reported previously based on measurements using fibre optic probes (Gu et al 2003). For the group of tumours in this study, mean baseline pO\textsubscript{2} did not provide a good indication of response to hyperoxic gas (\Delta pO\textsubscript{2} or pO\textsubscript{2max}). However, considering the fate of individual voxels in separate tumours with respect to intervention, strong linear correlation was observed in some tumours (tumour no 3, 4, 6 and 9) between initial mean baseline pO\textsubscript{2} and the maximum pO\textsubscript{2} at the same location (voxel) in response to carbogen or oxygen breathing consistent with the results of Song et al (2002). Other tumours showed no correlation.

The rate of pO\textsubscript{2} response to either gas was similar (figure 6), but decrease upon return to air was significantly faster in the case of oxygen, but not carbogen. While the relationships between \Delta pO\textsubscript{2} and \Delta [HbO\textsubscript{2}] are not obvious, there exists a significant correlation between \( f_1/f_2 \) and mean pO\textsubscript{2} values achieved with hyperoxic gas intervention. Most \( f_1/f_2 \) values are between 5 and 60 (figure 7(B)), implying that the blood perfusion rate in the well-perfused region is much higher than that from the poorly perfused region. Not surprisingly the higher the ratio of well perfused to poorly perfused regions, the higher the mean pO\textsubscript{2} values achieved by the hyperoxic gas interventions.

Previous studies have demonstrated that tumour tissue oxygenation could be strongly affected by changes in tumour blood flow measured locally by laser Doppler flowmetry (Vaupel et al 1994). It was also reported that spontaneous fluctuations in flow and perivascular pO\textsubscript{2} are correlated at the micro-regional level (Kimura et al 1996, Braun et al 1999).

In our study, we are able to derive the perfusion rate ratios between the well perfused and poorly perfused regions from the haemoglobin concentration responses to the intervention measured by NIRS. We found that there was a significant correlation between \( d(pO_2)/dt \) and \( A_1/\tau_1 (f_1, \text{proportional to } A_1/\tau_1) \), but not \( d(pO_2)/dt \) and \( A_2/\tau_2 (f_2, \text{proportional to } A_2/\tau_2) \), provided that the pO\textsubscript{2} readings were selected from well-oxygenated or responsive voxels (figure 8). This linear correlation suggests that the rate of change in pO\textsubscript{2} is closely related to the perfusion rate in the well-perfused region, \( f_1 \). In other words, the dynamic changes in pO\textsubscript{2} of those regions responsive to hyperoxic gas intervention may be attributed to fast tumour vascular perfusion, rather than to the slow perfusion in tumour vasculature. We believe these results provide a valuable association between tumour vascular oxygenation and tumour pO\textsubscript{2} determined simultaneously by the optical and NMR measurements.

A goal had been to develop a low-cost, simple, fast surrogate measurement of pO\textsubscript{2} based on NIRS of the oxygenation status of endogenous haemoglobin. Both figures 7(B) and 8 suggest that there exist linear relationships of pO\textsubscript{2} with the NIRS measurable parameters \( f_1/f_2 \) and with \( A_1/\tau_1 \). However, the correlation between \( \Delta pO_2 \) and \( \Delta [HbO_2] \) (figure 7(A)) seems to be more complex with a separation of the tumours into two groups. It may be noteworthy that the majority (6 of 7) of the tumours associated with the correlation on the left-hand side of the graph had a high initial global pO\textsubscript{2} (i.e., mean baseline pO\textsubscript{2} > 5 Torr), whereas 2 out of 3 of the tumours on the right had low initial global pO\textsubscript{2}. A possible interpretation relates to the shape of the haemoglobin–oxygen dissociation curve (the Hill curve). A given pO\textsubscript{2} response can correspond to different changes in haemoglobin saturation depending on where the change occurs on the Hill curve. At lower initial pO\textsubscript{2} there may be a substantial \( \Delta pO_2 \) with little \( \Delta [HbO_2] \). By contrast, at higher initial pO\textsubscript{2} the same increase in pO\textsubscript{2} could produce a greater effect on the saturation. While \( \Delta [HbO_2] \) is proportional to the change of SO\textsubscript{2} (with the assumption of little change in total haemoglobin concentration during intervention), two subpopulations of tumours in the relationship between \( \Delta pO_2 \) and \( \Delta [HbO_2] \) could be the result of different initial pO\textsubscript{2}. Similarly, two subpopulations of tumours were observed in the relationship between initial SO\textsubscript{2} and the carbogen-induced change in saturation in the study of Hull et al (1999). Such an effect also confounds the direct correlation of BOLD MRI response...

A major concern is tumour heterogeneity, as recognized throughout the literature and shown here by $^{19}$F MRI (figure 4). Indeed, we have obtained some preliminary data using a single NIR source and three detectors placed on various regions across a tumour (Kim et al 2003a), showing that each region of the tumour responded differently to hyperoxic gas, in terms of the extent and rate, indicating the heterogeneity of tumour vasculature. Spatial discrimination will be even more critical, if such studies are transferred to human breast cancer, where the tumour is surrounded by normal tissue (Brooksby et al 2003). Nevertheless, we believe this haemodynamic model and correlation between tumour vascular oxygenation and pO$_2$ provides valuable insight into the tumour compartment of such a mixed system and explores dynamic signatures of breast tumours, which could, in turn, enhance/assist human breast cancer diagnosis and prognosis.

In summary, by studying tumour vascular oxygenation concomitantly with changes in tumour oxygen tension, we found several significant correlations between rates and magnitudes of vascular and tissue responses. This study also demonstrates the feasibility of conducting simultaneous NIRS and MRI oximetry. We believe the correlation of tumour vascular oxygenation and tumour tissue pO$_2$ can provide valuable insights into tumour pathophysiology and response to interventions.

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Noninvasive Monitoring by Near-infrared Spectroscopy

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ABSTRACT

The goal of this study was to investigate the effect of photothermal laser irradiation on rat breast tumor (DMBA-4) vascular contents. An 805-nm diode laser was used in our experiment with a power density ranging from 0.32 to 1.27 W/cm². The dynamic changes of oxygenated hemoglobin and total hemoglobin concentrations, \(\Delta[HbO_2]\) and \(\Delta[Hb]_{total}\) in rat tumors during photothermal irradiation were noninvasively monitored by a near-infrared spectroscopy system. A multichannel thermal detection system was also used simultaneously to record temperatures at different locations within the tumors. Our experimental results showed that: (1) photothermal irradiation did have the ability to induce hyperthermic effects inside the rat breast tumors in a single exponential trend; (2) the significant changes \((P < 0.005)\) of \(\Delta[HbO_2]\) and \(\Delta[Hb]_{total}\) in response to a low dosage of laser irradiation (0.32 W/cm²) have a single exponential increasing trend, similar to that seen in the tumor interior temperature; and (3) the increase in magnitude of \(\Delta[HbO_2]\) is nearly two times greater than that of \(\Delta[Hb]_{total}\), suggesting that phototherapy may enhance tumor vascular oxygenation. The last observation may be important to reveal the hidden mechanism of photothermal irradiation on cancers, leading to improvement of tumor treatment efficiency.

INTRODUCTION

Laser phototherapy, a nonsurgical modality for cancer treatment, is gaining widespread acceptance because of precise energy delivery into the tumor tissue (1–3). The laser energy delivered to the targeted tumors can induce localized photomechanical, photochemical and photothermal reactions, thus killing tumor cells. In general, photochemical reactions may cause the change of chemical bonds and form toxic radicals, such as release of singlet oxygen, leading to the death of organized tissues. Photomechanical reactions may induce tissue stress, resulting in the disruption of tissue cells and ejection of material. Photothermal reactions may induce hyperthermia and coagulation, causing cell destruction (4). In particular, thermal cytotoxicity effects are found to be more profound under acidic (low pH) conditions, which are often present in poorly oxygenated tissues (5–7), such as in tumors. Many research studies have reported the existence of acute/chronic hypoxic regions within the majority of solid tumors (8–10). As a result, cytotoxicity induced by selective photothermal irradiation can be more effective in tumor tissue than in normal tissue. To further enhance the desirable photothermal effects, selective absorbers are often used in the targeted tumor tissue along with laser irradiation to cause selective and localized photon–tissue interactions (11,12). In addition, administration of an immunoadjuvant has been proved to increase systemic cancer cure and long-term resistance to cancer of the same origin, when combined with the selective photomechanical treatment (13,14). Chen et al. have developed a laser immunotherapy by combining laser irradiation, a laser-absorbing dye and an immunoadjuvant to improve cancer therapy efficiency (11,12,15,16). The previous experimental results have demonstrated that this unique approach has positive effects on both primary and metastatic tumors.

Although the underlying mechanism of the laser irradiation has been intensively studied (17–20), the hemodynamic and physiological properties inside the tumors during phototherapy are poorly understood. Measuring the reduction of tumor size and survival rates is the current practice to evaluate the therapeutic outcome of therapy. Obviously, noninvasive, real-time monitoring of tumor hemodynamic characteristics before, during and after therapy is highly desirable. Such a technique may help reveal the tumor physiology and therapeutic process caused by the therapy, providing treatment progresses and guidance to optimize the light dosimetry so as to improve the therapeutic outcome. Near-infrared spectroscopy (NIRS) has been demonstrated in our recent studies to be such a noninvasive means in monitoring tumor vascular oxygenation during therapeutic interventions (21–23). In this study, we investigated the effects of photothermal therapy on tumor vascular contents and tumor temperature. An 805-nm diode laser was used for photothermal irradiation of mammary tumors in rats. The dynamic changes caused by the laser irradiation in oxygenated, deoxygenated and total hemoglobin concentrations, \(\Delta[HbO_2]\), \(\Delta[Hb]_{total}\), and \(\Delta[Hb]_{total}\), in the tumor vasculature were...
The changes of oxygenated, deoxygenated and total hemoglobin concentration, $\Delta[HbO_2], \Delta[Hb]$ and $\Delta[Hb]_{total}$ in tumor tissue caused by laser irradiation were determined by a near-infrared, homodyne, frequency-domain spectroscopy system (NIM, Philadelphia, PA), which has been described in detail elsewhere (21–23). Briefly, the amplitude-modulated light at 140 MHz at wavelengths of 785 nm and 805 nm illuminated the tumor surface through a light delivery fiber bundle with output powers of 9 mW and 11 mW at 785 nm and 758 nm wavelengths, respectively, measured at the tip of the bundle by an optical power meter. The 805-nm laser light was vertically focused by a 0.6372 numerical aperture (NA) objective lens to create a 1-cm beam diameter for photothermal

monitored by an NIRS system. A multichannel, thermal monitor was also used simultaneously to record the temperatures at three different locations inside the breast tumors. The correlations between the optical irradiation dosages, tumor temperatures, and tumor vascular contents were studied. The dynamic features of tumor temperature and vascular oxygenation were quantified to reveal the dynamic effects within the tumors under the laser irradiation.

**Materials and Methods**

NIRS. The changes of oxygenated, deoxygenated and total hemoglobin concentrations, $\Delta[HbO_2], \Delta[Hb]$ and $\Delta[Hb]_{total}$, in tumor tissue caused by laser irradiation were determined by a near-infrared, homodyne, frequency-domain spectroscopy system (NIM, Philadelphia, PA), which has been described in detail elsewhere (21–23). Briefly, the amplitude-modulated light at 140 MHz at wavelengths of 785 nm and 805 nm illuminated the tumor surface through a light delivery fiber bundle with output powers of 9 mW and 11 mW at 785 nm and 758 nm wavelengths, respectively, measured at the tip of the bundle by an optical power meter. The experimental setup is shown in Fig. 1. The 805-nm laser light was vertically applied on the tumor surface. The delivering and detecting fiber bundles of the NIRS system were placed horizontally and in good contact with the surface of the tumors in a transmittance mode, without compressing the tumors. The diffused light through the tumor tissue was collected and filtered by a sharp low-pass optical filter, which cut off the optical signal above 805 nm from the laser irradiation, and then propagated into a photomultiplier tube (PMT) through the detecting fiber bundle. The signal detected by the PMT was demodulated through an in-phase and quadrature-phase (IQD) circuit, and the amplitude and phase of the signal were recorded.

Algorithm for calculations of $\Delta[HbO_2], \Delta[Hb]$ and $\Delta[Hb]_{total}$: On the basis of the Beer-Lambert law, light attenuation, represented by optical density (OD), through a sample is proportional to the concentration of hemoglobin inside the sample (24,25).

$$\text{OD} = \log(A_0/A) = \text{scf}$$

where $A_0$ and $A$ are light intensities of the incident and transmitted light, respectively; $c$ is the extinction coefficient of hemoglobin; $s$ is the concentration of hemoglobin, and $l$ is the length of light path through the measured sample. When the measured sample has a mixture of oxygenated and deoxygenated hemoglobin, Eq. (1) can be further evolved to (26–30).

$$\text{OD}(s) = \log([Hb][Hb] + [HbO_2][HbO_2])$$

where $D(s)$ is the optical density at wavelength $\lambda$, $\epsilon(Hb)$ and $\epsilon(HbO_2)$ are the extinction coefficients at wavelength $\lambda$ for molar concentrations of free oxygenated hemoglobin, [Hb], and oxygenated hemoglobin, [HbO2], respectively, assuming ferrichemoglobin is minimal. By using two wavelengths, both [HbO2] and [Hb] can be determined by measuring the OD values at the two specific wavelengths, provided that the values for $\epsilon(Hb)$ and $\epsilon(HbO_2)$ are known:

$$\begin{align*}
\Delta[HbO_2] & = [HbO_2](\lambda_1) - [HbO_2](\lambda_2) \\
\Delta[Hb] & = [Hb](\lambda_1) - [Hb](\lambda_2)
\end{align*}$$

$$\begin{align*}
\Delta[Hb]_{total} & = [Hb]_{total}(\lambda_1) - [Hb]_{total}(\lambda_2)
\end{align*}$$

It follows that changes in [Hb] and [HbO2] can be consequently given as:

$$\Delta[HbO_2] = \frac{\epsilon(HbO_2)\Delta[Hb]}{\epsilon(Hb)}$$

where $\epsilon(D(s))$ represents a change in optical density at the specific wavelength, $\lambda$, and equals $\log(A_0/A)$. $A_0$ and $A$ correspond to light intensities measured under the baseline and transient conditions.

Note that in principle, $l$ represents the optical path length between the source and detector. Whereas $l$ is simply the physical separation, $d$, between the source and detector through a nonscattering medium, exact quantification of $l$ for an intact tissue or organ is complex because of light scattering in tissue. Since $l$ is in proportion to the separation, $d$, we can associate $l$ to $d$ as $l = DPF \cdot d$, where $DPF$ is a differential path length factor to account for light scattering. It has been well accepted that together with $DPF$, Eq. (2) can be treated as modified Beer-Lambert law, and consequently, Eqs. (5) and (6) can be correctly used to quantify changes in [Hb] and [HbO2] in highly scattering media (29,30), as an intact tissue or organs.

To be consistent with our previous work, we adopted in this paper the values published by Zijlstra et al. (22). We had to interpolate the values at the two wavelengths used in our study, followed by certain corrections due to the interpolation errors by phantom calibration measurements (22). Specifically, we included two factors, $\beta_1$ and $\beta_2$, for the calibrated algorithm, as given below:

$$\begin{align*}
\Delta[HbO_2] & = \frac{\epsilon(HbO_2)\Delta[Hb]}{\epsilon(Hb)} - \frac{\epsilon(HbO_2)\Delta[Hb]}{\epsilon(Hb)} \\
\Delta[Hb] & = \frac{\epsilon(Hb)\Delta[Hb]}{\epsilon(Hb)} - \frac{\epsilon(Hb)\Delta[Hb]}{\epsilon(Hb)}
\end{align*}$$

$$\begin{align*}
\Delta[Hb]_{total} & = \frac{\epsilon(HbO_2)\Delta[Hb]}{\epsilon(Hb)} - \frac{\epsilon(HbO_2)\Delta[Hb]}{\epsilon(Hb)}
\end{align*}$$

where $\epsilon(D(s)) = 1.418, \epsilon(D(s)) = 0.672, \epsilon(D(s)) = 1.111, and \epsilon(D(s)) = 0.706$, all in mM cm$^{-1}$. Note that a factor of four has been multiplied for each of the $\epsilon(s)$ at the respective wavelengths to account for light absorption from four hemoglobin molecules (34), since the extinction coefficients published in the field of biochemistry were expressed on a heme basis (24–26). Furthermore, we have used $\beta_1 = 1.05$ and $\beta_2 = 0.9035$ according to our phantom study (22). After substituting all of the parameters into Eqs. (7) and (8), we have arrived at the final equations to quantify changes in hemoglobin concentration:

$$\begin{align*}
\Delta[HbO_2] & = -2.685 \cdot DPF785 \cdot \Delta[Hb]_{total} + 3.742 \cdot DPF785 \cdot \Delta[Hb] + 1.974 \cdot DPF785 \\
\Delta[Hb] & = -2.238 \cdot DPF785 \cdot \Delta[Hb]_{total} - 1.683 \cdot DPF785 \\
\Delta[Hb]_{total} & = \Delta[HbO_2] + \Delta[Hb]
\end{align*}$$

$$\begin{align*}
\Delta[HbO_2] & = -0.42 \cdot DPF785 \cdot \Delta[HbO_2] + 2.06 \cdot DPF785 \cdot \Delta[Hb]
\end{align*}$$

Notice that the units for $\Delta[HbO_2], \Delta[Hb]$ and $\Delta[Hb]_{total}$ are mM; however, the values obtained from Eqs. (9) to (11) are scaled by a factor of
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DPP. Since DPP is so far an unknown parameter for tumors, we include it within the unit as DPP.

Laser photothermal irradiation system. The system used for the photothermal therapy consisted of an NIR diode laser, DiodMed 25 (DiodMed, UK), with an emitting peak wavelength at 805 nm and a maximum power output of 25 W. The laser light was coupled into an optical fiber, fitted with a microlens at the tip (Pioneer Optics, Windsor Locks, CT) to ensure a uniform beam density, and delivered onto the tumor surface. The laser beam diameter projected on the tumor surface was set as 2 cm in all the measurements by using the visible aiming light in the laser system. After the beam diameter was adjusted, the aiming light was turned off during NIR measurements.

Multi-channel thermal monitor. A multi-channel thermal monitor (Omega, Stamford, CT), incorporating an OM-700 Omega engineering data acquisition and control unit, was used simultaneously with the NIRS observation to monitor the internal temperatures at several locations within the tumors. Specifically, three thermal probes were inserted at the top (immediately below the overlaying skin), middle, and bottom positions of the tumors, and one probe was located outside the tumor for room temperature recording. The data of each probe were recorded continuously, and the middle thermal probe recorded the internal tumor temperature near the center of the optical field of NIRS.

Animal model. The transplantable, metastatic mammary tumor cells (DIMBA-4) (33–35) were implanted in one of the inguinal fat pads of female White Rat hairless (Harlan Sprague Dawley, Indianapolis, IN). Once the tumors reached 0.5 to 0.8 cm in diameter, the rats were anesthetized for the experiments. Hairs around tumors were removed for better probe contact in the NIRS measurement and for more efficient tumor exposition to the laser irradiation. The tumor diameters along the three major orthogonal axes (a, b, c) were measured to determine the volume of the tumors by using an ellipsoid approximation with the formula of $V = \frac{4}{3}\pi abc$.

In this study, 16 rat breast tumors were used in two different treatment protocols. Ten rat tumors were first exposed to a single laser irradiation with a laser power of 0.32 W/cm$^2$ (1 W laser power in a 2-cm-diameter beam) for 10 min. Three or four days later, four of the treated tumors, along with other six untreated tumors, were exposed to repeated laser irradiations with a power density of 0.32, 0.64, 0.96 and 1.27 W/cm$^2$ (1 W, 2 W, 3 W, and 4 W in 2-cm-diameter beam) for 10 min with a recovery period of 10 min between each treatment. Tumor temperature, tumor vascular [HbO$_2$], and [Hb]$_{total}$ were measured simultaneously in all the experiments.

Statistical analysis. A Student’s t-test was applied in data analysis to determine whether the paired samples were significantly different. We considered $P < 0.05$ as the criteria for significant difference between the two groups. Standard deviation (mean ± sd) was usually used to express the measured data and their dispersions.

RESULTS

Dynamic responses of [HbO$_2$], [Hb]$_{total}$ and temperature to photothermal treatment

Using the experimental setup shown in Fig. 1 and Eqs. (9) and (11), tumor vascular [HbO$_2$], [Hb]$_{total}$ and internal temperatures were recorded simultaneously. Figure 2 shows the temporal profiles of [HbO$_2$], [Hb]$_{total}$ and the internal temperatures of a rat tumor under photothermal irradiation with a laser power density of 0.52 W/cm$^2$. The experimental data consisted of four temporal periods, as labeled in the Fig. In period 1, without irradiation, the thermal probes recorded the baseline temperatures at three different locations inside the tumor for about 6 min. In period 2, the NIRS system was turned on while the thermal recording continued, and the recorded thermal readings showed no obvious perturbation from the NIRS. After the NIRS signals were stabilized, a 10-min laser irradiation started. During the laser treatment, the tumor temperatures, [HbO$_2$], and [Hb]$_{total}$ showed significant elevation (period 3 in Fig. 2a). Tumor temperatures increased significantly ($P < 0.0001$) with the irradiation. The tumor volumes near the tumor surface had more significant thermal effects: the temperature at the top (T-top) varied from 25.3 ± 0.1°C to 30.3 ± 0.1°C (mean ± sd), while the temperatures at the middle and bottom (T-middle and T-bottom) of the tumor were elevated from 26.1 ± 0.1°C to 29.8 ± 0.1°C and from 27.5 ± 0.1°C to 28.9 ± 0.1°C, respectively. The maximal changes in T-top, T-middle and T-bottom caused by the laser irradiation were 5.8 ± 0.1°C, 3.7 ± 0.1°C and 1.4 ± 0.1°C, respectively. Similarly, both [HbO$_2$] and [Hb]$_{total}$ displayed significant increases over the entire irradiation period ($P < 0.0001$, with the mean elevations of 0.04 ± 0.01 mM/DPP and 0.02 ± 0.01 mM/DPP, respectively). Notice that the maximal values of [HbO$_2$] and [Hb]$_{total}$ shown in Fig. 2a were ~0.1 and 0.02 mM/DPP.

When the laser beam was turned off in period 4, [HbO$_2$] and [Hb]$_{total}$ and temperatures all decreased, with the temperatures reaching the baselines quickly and [HbO$_2$] and [Hb]$_{total}$ declining more gradually and not quite reaching the baselines within the 10-min recovery period.

To quantify the dynamic behaviors observed in Fig. 2a, single-exponential functions were applied to T-top, T-middle, [HbO$_2$], and [Hb]$_{total}$ on both rising and falling periods, as shown in Fig. 2b,c. The time constants obtained from such fittings reveal the dynamic responses of signals to the initiation and termination of photothermal irradiation. As shown in Fig. 2b, T-top (time constant of 4.5 ± 0.5 min) has a faster response to laser irradiation than T-middle (time constant at 14.4 ± 0.6 min). The time constants for [HbO$_2$] and [Hb]$_{total}$ on both rising and falling periods, respectively, are shown in Fig. 2c. It is clearly seen that the dynamic thermal responses of the tumor to laser irradiation and to the termination of laser irradiation are well matched both at the tumor surface and within the tumor. However, the time constants for [HbO$_2$] and [Hb]$_{total}$ to return to the baseline are much longer in the falling period, with 18.5 ± 0.5 min and 24.3 ± 0.8 min, respectively, also shown in Fig. 2c.

Similar protocols and measurements were performed on the other nine tumors. Figure 3 shows the average increases of the temperature at different locations. Temperatures near the tumor surface have maximal increases, whereas local temperatures near the bottom of the tumors have minimal increases observed from all the 10 tumors. The average elevations for T-top and T-bottom are 7 ± 3°C and 3 ± 2°C, respectively. The thermal readings from the middle position (T-middle) represent the average temperatures in the tumor volumes probed by the NIRS beams, and the average increase in T-middle for the 10 rats is 5 ± 2°C. The statistical analysis indicates significant differences between T-top, T-middle, and T-bottom ($P < 0.05$).

To investigate dynamic behaviors of the tumors in response to the initiation and termination of laser irradiation, the time constants of T-top, T-middle, [HbO$_2$], and [Hb]$_{total}$ obtained from the rising and falling periods are summarized in Table 1. The results demonstrate several points: (1) an average dynamic change in T-top is significantly ($P < 0.01$) faster than that in T-middle in the rising period, with a time constant of 3.7 ± 1.1 min for T-top and 6.4 ± 3.2 min for T-middle. A similar significant difference ($P < 0.01$) also holds in the falling period with a time constant of 3.8 ± 1.2 min for T-top and 6.6 ± 2.7 min for T-middle, respectively. (2) The dynamic responses of [HbO$_2$] and [Hb]$_{total}$ and T-middle have no significant differences during the initiation of photothermal irradiation ($P > 0.4$). (3) The average time constant of T-top in the rising period is similar ($P > 0.9$) to that in the falling period, and so is that of T-middle ($P > 0.7$). However, (4) the dynamic response of...
Figure 2. (a) Temporal profiles of the changes in tumor oxygenation and total hemoglobin concentrations, Δ[HbO2] and Δ[Hb]total, and tumor temperatures during photothermal intervention in a representative DMBA-4 rat breast tumor (2.4 cm3). The profile is divided into four periods, and the laser irradiation was applied only in period 3. (b) Respective single-exponential curve fittings for the rising period of T-top, T-middle, Δ[HbO2] and Δ[Hb]total displayed in (a). Single exponential fitting yields: T-top = 5.76*1 - exp[-(t - 14)](4.85) + 24.95 with R = 0.99; T-middle = 3.83*(1 - exp[-(t - 14)](4.38)) + 25.98 with R = 0.99; Δ[HbO2] = 0.038*(1 - exp[-(t - 14)](0.95)) + 0.101 with R = 0.99; Δ[Hb]total = 0.044*(1 - exp[-(t - 14)](1.10)) + 0.012 with R = 0.98. (c) Respective single-exponential curve fittings for the falling period of T-top, T-middle, Δ[HbO2] and Δ[Hb]total displayed in (a). Single exponential fitting yields: T-top = -0.56*(1 - exp[-(t - 14)](1.43)) + 24.95 with R = 0.99; T-middle = -0.56*(1 - exp[-(t - 14)](1.43)) + 24.95 with R = 0.99; Δ[HbO2] = 0.038*(1 - exp[-(t - 14)](0.95)) + 0.101 with R = 0.99; Δ[Hb]total = 0.044*(1 - exp[-(t - 14)](1.10)) + 0.012 with R = 0.98.

Effect of the optical power density on tumor Δ[HbO2], Δ[Hb]total and temperatures

Repeated laser irradiation was applied to the 10 tumors, 4 of which were used in the previous single-irradiation treatment 3 or 4 days earlier. Figure 5a shows the profiles of the tumor vascular Δ[HbO2], Δ[Hb]total and tumor temperatures in response to irradiations under power densities of 0.32, 0.64, 0.96 and 1.27 W/cm2, with an irradiation period of 10 min and a recovery period of 10 min between each treatment. Tumor temperatures (T-top, T-middle and T-bottom) increased significantly (P < 0.01) from the baseline to different maximum values under different laser powers. The maximal changes in temperature in the middle of the tumor (T-middle) due to consecutive irradiation displayed a strong linear dependence on the laser power density, as shown in Fig. 5b. Under relatively low laser power irradiations (0.32, 0.64, 0.96 W/cm2), both Δ[HbO2] and Δ[Hb]total increased significantly (P < 0.0001) with respect to the baselines, and they both declined significantly under a high laser power irradiation (1.27 W/cm2), as shown in

Δ[HbO2] in the falling period (a time constant of 28 ± 2.2 min) is significantly slower (P < 0.01) than that in the rising period (a time constant of 5.3 ± 2.8 min), and so is Δ[Hb]total response, with a time constant of 6.2 ± 3.7 min and 41 ± 32 min for the rising and falling periods, respectively (P < 0.01).

Furthermore, although Δ[HbO2] and Δ[Hb]total have similar dynamic trends in response to photothermolysis, a difference between their magnitudes exists. A strong correlation (R² = 0.95) between Δ[HbO2] and Δ[Hb]total has been observed, as shown in Fig. 4, where the amplitude in Δ[HbO2] is nearly twice that of Δ[Hb]total for all 10 tumors (with a slope of the fitted curve being 0.54).

Figure 3. Average changes in temperature from the 10 rats for T-top, T-middle and T-bottom.
(Fig. 5a), we had also taken a direct reading for 
\[ \text{W/cm}^2 \] 3 or 4 days before, the slopes of the linear regression for 
and nonpretreated with a single low-power irradiation (0.32 
\[ \text{D} \]
\[ i.e. \]
\[ \text{groups} \], each of the tumors shown in Fig. 6a were classified into two 
\[ \text{P} \]
\[ 5 \]
\[ (\text{test}) \] showed that there is no significant difference 
\[ \text{R} \]
\[ a \text{ correlation of } 0.91 \] for all the cases and having an average 
\[ \text{D} \]
\[ \text{middle increase linearly with the optical radiation dosages, with } \]
\[ \text{SD}^* \]
\[ 5 \]
\[ \text{average } 3.7 \]
\[ \text{SD}^* \]
\[ 1.1 \]
\[ \text{standard deviation.} \]
\[ \text{Tumor size(cm}^3\text{)} \]
\[ 2.4 \]
\[ 2.5 \]
\[ 0.68 \]
\[ 0.53 \]
\[ 1.2 \]
\[ 3.3 \]
\[ 1.9 \]
\[ 3.4 \]
\[ 2.1 \]
\[ 3.0 \]
\[ \text{Average } 3.7 \]
\[ \text{SD}^* \]
\[ 1.1 \]
\[ \text{Time constants (minutes) of } T\text{-top, } T\text{-middle, } \]
\[ \text{Rising part (in response to irradiation) Falling part (in response to termination) } \]
\[ T\text{-top } \]
\[ T\text{-middle } \]
\[ \Delta[HbO_2] \]
\[ \Delta[Hb]_{\text{total}} \]
\[ \text{SD}^* \]
\[ 0.53 \]
\[ 4.61 \]
\[ 7.62 \]
\[ 1.84 \]
\[ 1.91 \]
\[ 4.61 \]
\[ 0.68 \]
\[ 4.83 \]
\[ 4.85 \]
\[ 1.76 \]
\[ 1.66 \]
\[ 0.68 \]
\[ 3.28 \]
\[ 5.17 \]
\[ 6.9 \]
\[ 9.8 \]
\[ 1.2 \]
\[ 3.90 \]
\[ 6.65 \]
\[ 7.34 \]
\[ 7.83 \]
\[ 3.58 \]
\[ 4.45 \]
\[ 11.3 \]
\[ 29.9 \]
\[ 3.90 \]
\[ 1.89 \]
\[ 3.85 \]
\[ 10.0 \]
\[ 12.0 \]
\[ 1.30 \]
\[ 3.47 \]
\[ 20 \]
\[ 37 \]
\[ 3.90 \]
\[ 2.69 \]
\[ 3.13 \]
\[ 6.76 \]
\[ 7.81 \]
\[ 2.96 \]
\[ 4.58 \]
\[ 35 \]
\[ 45 \]
\[ 3.90 \]
\[ 3.25 \]
\[ 4.31 \]
\[ 7.32 \]
\[ 7.58 \]
\[ 4.52 \]
\[ 5.65 \]
\[ 23.5 \]
\[ 30 \]
\[ 3.90 \]
\[ 4.50 \]
\[ 7.80 \]
\[ 5.60 \]
\[ 8.40 \]
\[ 5.50 \]
\[ 8.30 \]
\[ 15.5 \]
\[ 23.4 \]
\[ 3.90 \]
\[ 1.1 \]
\[ 3.2 \]
\[ 2.8 \]
\[ 3.7 \]
\[ 1.2 \]
\[ 2.7 \]
\[ 22 \]
\[ 33 \]
\*SD = standard deviation.

Fig. 5b. Similar to the feature observed in single irradiation, 
\[ \Delta[HbO_2] \] values had greater magnitude than \[ \Delta[Hb]_{\text{total}} \], whereas 
their dynamic trends were consistent.

To investigate the accumulative effects on \[ \Delta[HbO_2] \] and 
\[ \Delta[Hb]_{\text{total}} \], from several thermal irradiations given 10 min apart 
(Fig. 5a), we had also taken a direct reading for \[ \Delta[HbO_2] \] and 
\[ \Delta[Hb]_{\text{total}} \] with a high laser power of 1.27 \text{ W/cm}^2 without any 
preirradiation. Figure 5c shows an example of this case, demon-
strating that \[ \Delta[HbO_2] \] indeed decreases after an initial increase for 
a few minutes, whereas \[ \Delta[Hb]_{\text{total}} \] has less trend to drop within 
the 10-min treatment. The similarity for the ratio of \[ \Delta[HbO_2] \] to 
\[ \Delta[Hb]_{\text{total}} \] close to a factor of 2 still exists.

Temperature data for all 10 tumors under different power 
irradiations are plotted in Fig. 6a. The maximum changes in T-
middle increase linearly with the optical radiation dosages, with 
a correlation of \[ R^2 > 0.91 \] for all the cases and having an average 
slope of \[ 13 \pm 3 \text{ (C/\text{W/cm}^2)} \]. To distinguish the tumors pretreated 
and nonpretreated with a single low-power irradiation (0.32 
\text{W/cm}^2) 3 or 4 days before, the slopes of the linear regression for 
each of the tumors shown in Fig. 6a were classified into two 
groups, i.e. (with preirradiation and with pretreatment (Fig. 6b). 
A Student’s \text{t}-test showed that there is no significant difference 
\[ (P = 0.95) \] between the two groups of slopes.

**DISCUSSION**

In this study, we have investigated vascular \[ \Delta[HbO_2] \], \[ \Delta[Hb]_{\text{total}} \] 
and internal temperature in response to photothermal therapy 
simultaneously in rat breast tumors by using an NIRS system 
and a thermal monitor, respectively. The results obtained in this study 
clearly demonstrate the compatibility of the NIRS system with the 
temperature monitoring system, without noticeable interference 
between the two systems (Fig. 2). The NIRS system offers a real-
time measurement of \[ \Delta[HbO_2] \] and \[ \Delta[Hb]_{\text{total}} \] in tumor vasculature 
within the optical field of the NIR probes, while the multichannel 
thermal monitor records the temperatures at specific locations 
within the tumors in real time. The compatibility of the two 
systems permits simultaneous determinations of thermal and 
vascular characteristics of treated tumors under photothermal 
therapy, providing valuable insight into the dynamic relationship 
between the tumor vascular contents and the thermal effect in 
treated tumor tissues.

Thermal treatment to tumors has been demonstrated to cause 
cellular cytotoxicity, and thus, to destroy the tumor cells (36–38). 
Laser irradiation applied in this study proves its ability to induce 
photothermal effects inside the rat breast tumors. The single 
exponential increase in tumor temperature obtained in our results 
indicates that an extended exposure period to the laser beam may 
gradually stabilize the tumor temperature at a certain level, 
depending on both the laser power density and the individual 
tumors (as seen in Figs. 2a and 5a). Manipulation of the laser 
power may help adjust the internal tumor temperature, resulting in 
optimal tissue destruction. Although a strong linear relation 
between the maximum temperature increase within the tumors 
and the irradiation power density has been observed for all the 
tumors, the actual dependence of tumor temperature on the laser 
power density does vary from tumor to tumor (Fig. 6). Such 
inter-tumor variability demonstrates the necessity of real-time 
monitoring for tumor temperature under photothermal therapy. 
Moreover, the tumor temperatures near the surface (T-top) respond 
to the photothermolysis more rapidly and with a greater increase 
than those within deeper tumor tissue (T-middle) (as seen in 
Figs. 2, 3, and 5a, and Table 1). Such large thermal effects can 
cause tissue damage at the tumor surface. To avoid such thermal damage,
intratumoral injection of selective absorbers, such as indocyanine green, has been used by Chen et al. to selectively manipulate the temperature within the tumor without severe damage at the tumor surface (11,12,39).

It is seen clearly in this study that tumor vascular contents, i.e. oxygenated and deoxygenated hemoglobin, are greatly affected by photothermal therapy. When the photothermal irradiation with a low power density (0.32 W/cm²) was applied to the breast tumors, both \( \Delta[HbO_2] \) and \( \Delta[Hb]_{\text{total}} \) increased significantly \((P, 0.005)\) in all the tumors (Figs. 2a, 4 and 5a). The dynamic behaviors of \( \Delta[HbO_2] \) and \( \Delta[Hb]_{\text{total}} \) in response to photothermal irradiation, quantified by the time constants, are in the same order as those of tumor interior temperature, T-middle, while their behaviors in response to the termination of photothermal irradiation are much slower than those of T-middle (Fig. 2 and Table 1).

In contrast to the lower laser power irradiation, higher laser power densities could cause interesting responses in tumor vascular contents. As seen in Fig. 5b,c, \( \Delta[HbO_2] \) started to decrease when the laser power approached a certain level, either with or without preirradiation. Intriguingly, although \( \Delta[HbO_2] \) has the same dynamic trend as \( \Delta[Hb]_{\text{total}} \) in response to photothermal therapy, the magnitude of \( \Delta[HbO_2] \) is more profound than that of \( \Delta[Hb]_{\text{total}} \) (Figs. 4, 5).

The analysis on the dynamic behaviors of tumor vascular contents and interior tumor temperature in response to photothermal therapy allows us to better understand the mechanism of photothermal therapy. The time constants of \( \Delta[HbO_2] \) as well as of \( \Delta[Hb]_{\text{total}} \) have the same order as those of interior tumor temperature. We may speculate that an increase in tumor temperature may lead to vessel dilation within tumor vasculature, and thus, resulting in an increase in blood inflow. Such an increase in blood inflow will give rise to an increase in \( \Delta[Hb]_{\text{total}} \) as well as \( \Delta[HbO_2] \).

Besides thermal effects, it is reasonable to expect other photoactivation processes involved in the laser irradiation. It is known that direct exposure of vascular tissue to visible light causes vasodilation (40), whereas the thermal effect from visible light is minor. Photoactivation has also been reported to cause
upregulation of nitric oxide, and thus, produce vasodilation (41–43). The increase of tumor blood contents due to laser irradiation observed in our study may be attributed to the vessel dilation induced by the direct effect of heating as well as photoactivation. Consistent with our observations, enhancement of both [HbO2]total and [Hb]total in response to laser irradiation indicated the existence of vessel dilation. The fact that the time constants of [HbO2]total and [Hb]total in response to laser irradiation increased with the time intervals between two repeated treatments, and affects the response of tumor to subsequent high irradiation; (5) the fact that the change in vascular oxygenation, which will be beneficial to some nonsurgical treatments, such as chemotherapy and radiotherapy (37).

In summary, changes in breast tumor temperature and vascular oxygenation have been simultaneously measured using a multi-channel thermal monitor and an NIRS system, while the tumors were under photothermal irradiation. The results have demonstrated that: (1) the compatibility of thermal monitoring system and the NIRS system permits simultaneous determinations of thermal and vascular characteristics of the treated tumors; (2) photoirradiation may increase the tumor vascular contents to 808-nm diode laser. Cancer Lett. 88, 15–19.


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Engineering in Genomics

extinction coefficients of hemoglobin for near-infrared spectroscopy of tissue

Jae G. Kim, Mengna Xia, and Hanli Liu

Extinction coefficients of hemoglobin have been studied for five decades by clinical chemists and biochemists, particularly for laboratory spectrophotometric measurements. A representative list of literature can be found in [1]–[12], and the studies on the temperature dependence of extinction coefficients of hemoglobin can be found in [13] and [14].

However, during the last ten to 15 years, near infrared spectroscopy (NIRS) and imaging for tissue vascular oxygenation, breast tumor detection, and functional brain imaging have been intensively developed for in vivo measurements by groups of physicists, biomedical engineers, and mathematicians. In the approach of NIRS, NIR light in the wavelength range of 650–900 nm is utilized to illuminate tissue in vivo, and the transmitted or reflected light through tissue is recorded for the quantification of hemoglobin concentrations of the measured tissue vasculature. In order to achieve mathematical conversion from the detected light intensity at different wavelengths to hemoglobin concentration, extinction coefficients of hemoglobin, ε, must be used.

While the engineers and physicists working in the NIR field have found the correct ε values to use, there has been controversy on what ε values should be used for in vivo NIRS in comparison with the conventional ε that most biochemists have used in the laboratories for in vitro measurements. The purpose of this article is to address this issue and help biomedical engineers and physicists gain a better understanding of ε to be used for NIRS and NIR imaging.

Spectrophotometric Measurements to Determine Hemoglobin Concentration

Hemoglobin is a molecule in the red blood cells that has a role of delivering oxygen to tissue cells. Hemoglobin is composed of four heme groups and a protein group, known as a globin. Historically, for spectrophotometric experiments, biological chemists and biochemists utilized Beer-Lambert’s law and developed the notation of absorbance to express light absorption as a function of hemoglobin concentration as given in [1]–[5]:

$$\text{OD} = \log(I_0/I) = \varepsilon c L$$  \hspace{1cm} (1)

where OD is the optical density, $I_0$ is the light intensity of incident light, $I$ is the light intensity of transmitted light, ε is the extinction coefficient of hemoglobin, c is the concentration of hemoglobin, and L is the length of light path through solution.

When the measured sample has a mixture of oxygenated and deoxygenated hemoglobin, (1) can be further expanded as [3]–[5],

$$\text{OD}^\lambda = [\varepsilon_{\text{Hb}}^\lambda [\text{Hb}] + \varepsilon_{\text{HbO}_2}^\lambda [\text{HbO}_2]]L$$  \hspace{1cm} (2)

where OD$^\lambda$ is the optical density or absorbance at wavelength $\lambda$ and $\varepsilon_{\text{Hb}}(\lambda)$ and $\varepsilon_{\text{HbO}_2}(\lambda)$ are the extinction coefficients at wavelength $\lambda$ for molar concentrations of deoxygenated hemoglobin, [Hb], and oxygenated hemoglobin, [HbO$_2$], respectively, assuming ferrihemoglobin is minimal. In some references, the light path, L, was taken as 1 cm without mentioning the definition of L each time [3]–[5]. Both [HbO$_2$] and [Hb] can be determined by measuring the light absorbance at the two specific wavelengths, provided that the values for $\varepsilon_{\text{Hb}}(\lambda)$ and $\varepsilon_{\text{HbO}_2}(\lambda)$ are known, as expressed below.

$$[\text{HbO}_2] = \frac{\varepsilon_{\text{Hb}}^\lambda \text{OD}^\lambda - \varepsilon_{\text{Hb}}^\lambda \text{OD}^\lambda}{L(\varepsilon_{\text{Hb}}^\lambda \varepsilon_{\text{HbO}_2}^\lambda - \varepsilon_{\text{Hb}}^\lambda \varepsilon_{\text{HbO}_2}^\lambda)}$$ \hspace{1cm} (3)

$$[\text{Hb}] = \frac{\varepsilon_{\text{HbO}_2}^\lambda \text{OD}^\lambda - \varepsilon_{\text{HbO}_2}^\lambda \text{OD}^\lambda}{L(\varepsilon_{\text{Hb}}^\lambda \varepsilon_{\text{HbO}_2}^\lambda - \varepsilon_{\text{Hb}}^\lambda \varepsilon_{\text{HbO}_2}^\lambda)}$$ \hspace{1cm} (4)

It follows that changes in [Hb] and [HbO$_2$] can be consequently given as

$$\Delta[\text{HbO}_2] = \frac{\varepsilon_{\text{Hb}}^\lambda \Delta \text{OD}^\lambda - \varepsilon_{\text{Hb}}^\lambda \Delta \text{OD}^\lambda}{L(\varepsilon_{\text{Hb}}^\lambda \varepsilon_{\text{HbO}_2}^\lambda - \varepsilon_{\text{Hb}}^\lambda \varepsilon_{\text{HbO}_2}^\lambda)}$$ \hspace{1cm} (5)

$$\Delta[\text{Hb}] = \frac{\varepsilon_{\text{HbO}_2}^\lambda \Delta \text{OD}^\lambda - \varepsilon_{\text{HbO}_2}^\lambda \Delta \text{OD}^\lambda}{L(\varepsilon_{\text{Hb}}^\lambda \varepsilon_{\text{HbO}_2}^\lambda - \varepsilon_{\text{Hb}}^\lambda \varepsilon_{\text{HbO}_2}^\lambda)}$$ \hspace{1cm} (6)

$$\Delta[\text{Hb}]_{\text{total}} = \Delta[\text{Hb}] + \Delta[\text{HbO}_2]$$ \hspace{1cm} (7)

where $\Delta\text{OD}^\lambda$ represents a change in optical density at the specific wavelength, $\lambda$, and equals log$(I_T/I_B)$. $I_B$ and $I_T$ correspond to light intensities measured under the baseline and transient conditions.

These spectrophotometric calculations seem straightforward mathematically and have been used for several decades by biochemists to quantify [Hb] and [HbO$_2$] in the laboratory measurements. However, close attention must be paid to the definition and accuracy of ε since biochemical details in obtaining ε
values give rise to the different quantification of hemoglobin concentration. In early publications on spectrophotometry of hemoglobin [3], it was clearly stated that all extinction coefficients were expressed on a heme basis, using a term of equivalent, where per equivalent of hemoglobin was assumed to be 66,800/4 or 16,700 gm, i.e., one-quarter of the molecular weight of the hemoglobin molecule [12]. While spectroscopic absorption measurements became a popular methodology for biochemists to quantify [Hb] and [HbO2] in laboratories [5], [6], [12] the notation of extinction coefficients being based on one heme group (or per equivalent) gradually has not been mentioned and has become conventionally understood by biological chemists for the last two to three decades.

Near-Infrared Spectroscopy Used to Determine Hemoglobin Concentration

For the last 15 or more years, significant research efforts have been conducted using NIRS for in vivo quantification of hemoglobin concentrations from intact living tissues. At the early stage of development, direct utilization of (3) and (4) was performed to obtain values or changes of oxygenated and deoxygenated hemoglobin concentration from living tissues in vivo [15]–[18], without particularly noticing that most of the $\varepsilon$ values published in biological literature were extinction coefficients for hemoglobin per equivalent (or per one heme of hemoglobin). Such confusion was propagated in some recent publications [19], including our own studies [20]–[22], where the $\varepsilon$ values given by Zijlstra et al. have been applied [7].

It gradually becomes clear to the authors that for NIRS of hemoglobin quantification, a factor of four needs to be multiplied by the $\varepsilon$ values published by the conventional biochemistry methods. This is a conversion factor to account for four hemes per hemoglobin molecule, which has been demonstrated by Mark Cope [10]. In this way, more meaningful results for concentrations of [Hb] and [HbO2] can be arrived from the NIR absorption measurements.

Furthermore, accurate determination of [Hb] and [HbO2], or $\Delta$[Hb] and $\Delta$[HbO2], is highly sensitive to the accuracy of $\varepsilon$ values, and intensive studies have been conducted over decades on how to determine accurate $\varepsilon$ values [6]–[8], [13]–[14]. The $\varepsilon$ values used in our earlier publications were obtained using data interpolation from Zijlstra’s $\varepsilon$ table [7]. However, such data interpolation for $\varepsilon$ at different wavelengths contained potential errors because of the nonlinearity of $\varepsilon$. Therefore, a calibration procedure was necessary and correction factors had to be applied in our earlier algorithm development [21]. On the other hand, Mark Cope has generated a complete $\varepsilon$ table at each wavelength between 650 and 999 nm, while taking four hemes into consideration. Such an $\varepsilon$ table has been recently well accepted and used [23]–[25].

Comparison of Published Extinction Coefficients for NIRS Given by Two Groups

Since Hoppe-Seyler first described the visible bands of oxyhemoglobin in 1862 [26], numerous reports on hemo-

![Fig. 1. Absorption spectra of oxy- and deoxyhemoglobin from Wray (9), Cope (10), and Zijlstra (7), (8).](image-url)
globin absorption spectra have appeared in the literature. However, only a few have tabular forms of hemoglobin extinction coefficients. In particular, two groups have reported tabular forms of hemoglobin extinction coefficients in a broad range of the NIR spectrum. Zijlstra et al. reported \( \varepsilon \) values for human blood [7], followed by another publication for updated human \( \varepsilon \) values with a comparison to those of rat blood [8]. Moreover, Cope has generated a complete \( \varepsilon \) table at each wavelength between 650 and 999 nm, while taking four hemes into account for in vivo NIRS measurements. Cope also compared his own experiments with other previous reports, finding that the isobestic point of hemoglobin is shifted to 798 ± 1.5 nm from 800 nm reported by Horecker [1], 805 nm by Barlow [27] and 815 nm by van Assendelft [28].

It is worthwhile to note that the determination of \( \varepsilon \) values has resulted in a range of accuracy claims, even for values reported from the same group. For example, according to Zijlstra et al., a value of \( \varepsilon_{\text{Hb}} \) at 560 nm in [29] was 12.72 (mM\(^{-1}\)cm\(^{-1}\)), while it was later changed to 13.09 (mM\(^{-1}\)cm\(^{-1}\)) in [7]; a value of \( \varepsilon_{\text{HbO}_2} \) at 630 nm was 0.11 (mM\(^{-1}\)cm\(^{-1}\)) as given in [7], while it was also reported as 0.14 (mM\(^{-1}\)cm\(^{-1}\)) in [8]. From Cope’s group, their published reports show that \( \varepsilon_{\text{Hb}} \) and \( \varepsilon_{\text{HbO}_2} \) at 752 nm are 1.574 (mM\(^{-1}\)cm\(^{-1}\)) and 0.608 (mM\(^{-1}\)cm\(^{-1}\)), respectively, as given in [9], while the corresponding \( \varepsilon \) values are 1.5938 (mM\(^{-1}\)cm\(^{-1}\)) and 0.5613 (mM\(^{-1}\)cm\(^{-1}\)) in [10]. A complete comparison of \( \varepsilon \) values based on [7]–[10] by Zijlstra et al., Wray et al., and Cope is plotted in Figure 1, showing the similarities and differences among the reported extinction coefficients of oxy- and deoxyhemoglobin. Notice that the values plotted from Zijlstra’s group have been multiplied by a factor of four to account for four hemes per hemoglobin molecule for in vivo measurements. It is obvious that certain variations among different sets of \( \varepsilon \) values exist, while the basic trends are the same.

Table 1, on the other hand, shows the tabular form of hemoglobin extinction coefficients from two groups. Extinction coefficients in the left two columns of \( \varepsilon_{\text{Hb}} \) and \( \varepsilon_{\text{HbO}_2} \) are from Zijlstra’s two reports [7], [8] and those in the third column of \( \varepsilon_{\text{Hb}} \) and \( \varepsilon_{\text{HbO}_2} \) are from Cope’s report [10]. Since the tabular form of extinction coefficients from Zijlstra does not have values of 758 and 785 nm, the extinction coefficients of 758 and 785 nm were obtained by linear interpolation between 750 and 775 nm and between 775 and 800 nm, respectively. The hemoglobin extinction coefficients from Cope were divided by four to be “per equivalent” so that they could be compared to those from Zijlstra et al. The differences listed in the last column are the differences between Cope’s \( \varepsilon \) values and the values from Zijlstra et al [8].

While the differences in \( \varepsilon \) values seem small among the published data, they can result in ~20% variation in quantification of hemoglobin concentration, i.e., \([\text{Hb}], [\text{HbO}_2],\) and \([\text{Hb}]_{\text{total}}\) for the NIRS determined values. The error range varies, depending on the actual wavelengths used. We are currently conducting more quantitative error analysis to demonstrate the importance of accurate \( \varepsilon \) for NIRS. Meanwhile, we wish in this report to help biomedical engineers better understand the extinction coefficients of hemoglobin and to further draw attention to the dependence of NIRS accuracy on the extinction coefficients of hemoglobin.

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COMAR Reports (continued from page 117)
Simultaneous monitoring of tumor vascular oxygenation and tissue oxygen tension under hyperbaric oxygen exposure

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Abstract: We demonstrate the ability to investigate breast tumor oxygen dynamics simultaneously by steady-state diffuse reflectance spectroscopy and FOXY oxygen sensor in response to hyperbaric oxygen intervention.

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1. Introduction
Tumor hypoxia has proven to have a prognostic impact in cancers and is associated with poor response to radiotherapy [1,2]. Hyperbaric oxygenation offers an approach to overcome tissue or tumor hypoxia. Several randomized studies investigating hyperbaric oxygenation as a radiosensitizer were performed 30-40 years ago [3-5]. However, hyperbaric oxygenation was largely abandoned. Recently, a few groups have developed and demonstrated a new strategy to combine hyperbaric oxygenation and radiotherapy, based on the finding that tumors maintained elevated oxygenation even after exposure to hyperbaric oxygen [6-8]. Accordingly, accurate evaluation of tumor oxygenation could be crucial for determination of radio-sensitivity. In our study, we applied two monitoring techniques simultaneously, i.e., steady-state diffuse reflectance spectroscopy (SSDRS) to monitor tumor vascular oxygenation and a multi-channel FOXY oxygen sensor to measure regional tumor oxygen tension when the rats were under hyperbaric oxygen exposure. The objective of study was to investigate breast tumor oxygen dynamics in response to hyperbaric oxygenation and to examine whether an improvement in tumor oxygenation is maintained after hyperbaric oxygenation.

2. Materials and Methods

2.1 Animal Preparation and experimental setup
Female Fischer 344 rats were used with subcutaneously growing mammary adenocarcinoma 13 762NF (originally obtained from DCT, NIH) on the dorsum of the thigh. When the tumors reached ~1 cm in diameter, the rats were anesthetized with ketamine hydrochloride (1.5ml; 100mg/ml; Aveco, Fort Dodge, IA) and xylazine by intraperitoneal injection. Tumor hair was trimmed for the ease of optical contact for transmitting NIR light and FOXY probe insertion. The rats were placed in the hyperbaric chamber on their sides, and then the probes of SSDRS and FOXY were fixed securely on the tumor. Tumor oxygenation parameters were measured simultaneously by SSDRS and FOXY during respiratory challenge with hyperbaric oxygen (HBO) or carbogen (HBCB).

During the experiments, the rats were exposed to hyperbaric oxygen in an acrylic hyperbaric chamber. The chamber was flushed with air for 15 min, followed by normobaric 100% oxygen or carbogen for 15 min, then followed by a pressure increase to 2 atmospheres absolute (HBO) for 30 min. The chamber pressure was then reduced to ambient, followed by a flushing with air. Gas exchange (air to oxygen/carbogen) was accomplished at an initial flow rate of approximately 15 l/min. Compression and decompression (from 1 to 2 atmosphere oxygen/carbogen, and 2 to 1 atmosphere oxygen/carbogen) required approximately 2 min.

2.2 Steady-state diffuse reflectance spectroscopy (SSDRS) for measuring changes in tumor vascular oxygenation ($\Delta$HbO$_2$)
The instrument used to acquire reflectance spectra from tumor tissue is a broadband diffuse reflectance spectrometer. Briefly, continuous wave (CW) light from a 20 W tungsten-halogen light source (HL-2000HP, ocean optics, FL) is coupled into a 2.6-mm core diameter fiber optic bundle, the distal end of which is placed in physical contact with the surface of the tumor. After being scattered in the tumor tissue, the transmitted light is collected by a 1-mm core
diameter detection fiber, the end of which is coupled to a hand-held spectrometer (USB2000, Ocean optics, FL). The broadband light diffuse spectrometer provides reflectance spectra from 400 to 900 nm.

According to the modified Beer-Lambert law, as given in Eqs. (1) and (2), changes of oxy- and deoxy-hemoglobin concentration, \( \Delta [HbO_2] \) and \( \Delta [Hb] \), can be derived from the measured amplitudes at two wavelengths (750nm and 830nm), by using extinction coefficients of oxy- and deoxy-hemoglobin published by Cope [9].

\[
\log\left(\frac{A_b}{A_t}\right)_{750} = (\varepsilon_{HbO_2} \Delta [Hb] + \varepsilon_{HbO_2} \Delta [HbO_2]) \cdot DPF \cdot d
\]

(1)

\[
\log\left(\frac{A_b}{A_t}\right)_{830} = (\varepsilon_{Hb} \Delta [Hb] + \varepsilon_{Hb} \Delta [HbO_2]) \cdot DPF \cdot d
\]

(2)

where \( A_b \) is the baseline amplitude, \( A_t \) is the transient amplitude during the intervention, and \( d \) is the direct source-detector separation. \( DPF \) (differential path-length factor) is a tissue-dependent parameter and defined as the ratio between the optical path length and the physical separation between the source and detector.

2.3 FOXY oxygen sensor for measuring oxygen tension of tumors (pO2)

Simultaneously, regional pO2 values in tumors were monitored by a multi-channel, fiber optic oxygen sensor (FOXY, Ocean Optics Inc, Dunedin, FL). Three fluorescence-quenched, optical fiber probes (AL300, tip diameter 410 \( \mu \)m) were inserted into different regions of the tumors. Light from a pulsed blue LED (475 nm) was coupled into one branch of a bifurcated optical fiber probe and propagated to the probe tip. The distal end of the probe is coated with a thin layer of a hydrophobic sol-gel material, in which oxygen-sensing ruthenium complex is effectively trapped. Illumination of the ruthenium complex causes fluorescence at ~600 nm. If the excited ruthenium complex encounters oxygen molecule, the excess energy is transferred to the oxygen molecule, thus quenching the fluorescence signal. The degree of quenching correlates with the oxygen concentration, pO2.

3. Results

![Fig.1 Time course of \( \Delta [HbO_2] \) and pO2 in response to respiratory challenge for a representative tumor, simultaneous measured by SSSDRS (thicker curve) and FOXY (thinner curves). The symbol “*” represents the time needed for decompression.](image)

Figure 1 shows simultaneous readings of changes in [HbO2] measured by SSSDRS and three pO2 traces in three regions of a breast tumor recorded by FOXY in response to respiratory challenge. This tumor showed a rapid response to oxygen intervention in normal pressure with a significant increase in \( \Delta [HbO_2] \). The three readings in pO2 displayed heterogeneous responses to normobaric oxygen inhalation. One region showed distinct improvement in pO2, while the other two regions had little or no improvement in pO2. When the oxygen was pressurized to 2 atmospheres, \( \Delta [HbO_2] \) had a further increase with an initial increasing rate less than that achieved with normobaric oxygen. However, pO2 readings in the three respective regions showed significant improvement under the hyperbaric oxygen condition. Returning to air from hyperbaric oxygen produced a gradual decline for both \( \Delta [HbO_2] \) and pO2. A strong linear correlation was observed between maximal \( \Delta pO_2 \) achieved with hyperbaric Oxygen/Carbogen intervention and that achieved at the same location with respect to normobaric oxygen/carbogen intervention in 11 tumors (Fig. 2).
Tumor $pO_2$ (average value of a group of six tumors with HB O) in air was $11.7 \pm 2.6$ mmHg, and it increased significantly during normobaric oxygen to $17.5 \pm 4$ mmHg ($p < 0.01$). Hyperbaric oxygenation resulted in a more significant increase in $pO_2$ than normobaric oxygenation ($p < 0.005$), as shown in Figure 3. Following hyperbaric oxygen, the $pO_2$ decrease to $21.5 \pm 4.6$ mmHg in 10 min, $16.9 \pm 3.1$ mmHg in 15 min, and then $14.3 \pm 2.4$ mmHg in 20 min. Tumor $pO_2$ values showed similar responses for a group of 5 tumors with hyperbaric carbogen intervention.

4. Discussion
This study shows that the two techniques, i.e., SSDRS and FOXY, are consistent and complementary with one another for tumor oxygenimetry. Our data demonstrates that hyperbaric oxygenation improves the tumor oxygenation better than normobaric oxygen. It also suggests that an improvement in tumor tissue oxygenation achieved by hyperbaric oxygenation may be maintained over 10-20 minutes even after hyperbaric oxygenation.

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6. References