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To cite this article: Anne L van de Ven et al 2013 New J. Phys. 15 055004

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Modeling of nanotherapeutics delivery based on tumor perfusion

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New Journal of Physics 15 (2013) 055004 (22pp)
Received 3 October 2012
Published 8 May 2013
Online at http://www.njp.org/
doi:10.1088/1367-2630/15/5/055004

Abstract. Heterogeneities in the perfusion of solid tumors prevent optimal delivery of nanotherapeutics. Clinical imaging protocols for obtaining patient-specific data have proven difficult to implement. It is challenging to determine which perfusion features hold greater prognostic value and to relate measurements to vessel structure and function. With the advent of systemically administered nanotherapeutics whose delivery is dependent on

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overcoming diffusive and convective barriers to transport, such knowledge is increasingly important. We describe a framework for the automated evaluation of vascular perfusion curves measured at the single vessel level. Primary tumor fragments, collected from triple-negative breast cancer patients and grown as xenografts in mice, were injected with fluorescence contrast and monitored using intravital microscopy. The time to arterial peak and venous delay, two features whose probability distributions were measured directly from time-series curves, were analyzed using a fuzzy c-mean supervised classifier in order to rank individual tumors according to their perfusion characteristics. The resulting rankings correlated inversely with experimental nanoparticle accumulation measurements, enabling the modeling of nanotherapeutics delivery without requiring any underlying assumptions about tissue structure or function, or heterogeneities contained therein. With additional calibration, these methodologies may enable the investigation of nanotherapeutics delivery strategies in a variety of tumor models.

Online supplementary data available from stacks.iop.org/NJP/15/055004/mmedia

Contents

1. Introduction
2. Materials and methods
   2.1. Silicon particle fabrication
   2.2. Animal models
   2.3. Intravital microscopy image acquisition
   2.4. Quantification of particle accumulation
   2.5. Quantification of tracer perfusion
   2.6. Perfusion classification
   2.7. Automated tumor ranking by fuzzy c-mean classification
   2.8. Model validation
3. Results
   3.1. Nanotherapeutics delivery is heterogeneous from tumor to tumor
   3.2. Quantification of tracer first-pass perfusion
   3.3. Identification of tissue classifiers
   3.4. Classification of tumor vascularity
4. Discussion
Acknowledgments
Appendix
References

1. Introduction

Tumor vasculature is characterized by structural abnormalities that produce spatial and temporal heterogeneities in blood flow. The vasculature lacks a regular hierarchical network of large proximal vessels feeding into successively smaller vessels; instead, vessel interconnections
are irregular in size and spacing \[1, 2\]. Endothelial cells lining these vessels have an altered morphology, pericytes (cells that support endothelial cells) are poorly attached or are absent, and the basement membrane is often abnormal. The resulting vessels are dilated, tortuous, saccular, and are vulnerable to collapse \[1, 2\]. The presence of fenestrations \[3–5\], combined with incomplete vascular walls \[5\], can yield localized regions of blood plasma leakage that alter macromolecule transport \[6, 7\] and increase interstitial pressure \[8\]. Collectively, these vascular abnormalities lead to regions of tumor tissue that are perfused poorly, intermittently or not at all \[9, 10\].

Non-uniformities in vascular perfusion hinder the transport of chemotherapeutics. The delivery of conventional chemotherapeutics relies on passive diffusion from the bloodstream into the tumor interstitium, and is therefore largely constrained by the drug circulation half-life, distance of travel and the pressure differential between the vasculature and tissue \[11\]. Nanotherapeutics, in which drugs are concentrated inside longer-circulating nanoscale delivery vehicles, have been developed to enhance drug accumulation within tumors. Early clinical successes, such as Doxil (liposome-encapsulated doxorubicin) and Abraxane (albumin-stabilized paclitaxel), rely on the enhanced permeation and retention (EPR) effect to passively accumulate in tumor fenestrations \[12\]. The overall efficacy of Doxil and other nanotherapeutics, however, remains variable due to patient-specific heterogeneities in tumor vascularization, interstitial pressure and drug retention \[8\]. Advances in multi-stage silicon nanovehicles \[13\] promise to overcome many of these transport barriers, since the physical properties of these particles (size, shape, surface chemistry, etc) can be modulated to improve tumor-specific accumulation in an EPR-independent manner \[14, 15\]. The greatest challenge to the delivery of such nanotherapeutics lies in optimizing particles to leverage transport conditions that are not conducive to conventional chemotherapeutics delivery \[16\].

Tumor perfusion remains poorly understood, particularly with respect to what conditions lead to effective or poor treatment. Attempts to characterize tumor perfusion using static data, such as the measurement of microvessel density from patient biopsies, have shown mixed prognostic capacity \[17, 18\]. Clinical imaging modalities capable of monitoring perfusion dynamically, such as magnetic resonance imaging (MRI) \[19\], computed tomography (CT) \[20\], positron emission tomography (PET) \[21\] and Doppler sonography \[22\], have been used to produce time-series images that enable pixel-by-pixel analysis of contrast kinetics within tumors. Parameters measured from the resulting ‘time–signal’ curves are placed in pharmacokinetic (PK) models in order to extrapolate information regarding vascular anatomy and physiology. Principal features derived using PK models include the blood flow velocity, blood volume and mean transit time. Numerous methods have been proposed to extract these features in human tissues \[23–31\]. While the prognostic capacity of such an approach remains to be determined, MRI \[32\], CT \[33\] and PET \[34\] have demonstrated that tumor transport plays a role in treatment response, and that persistence of unfavorable perfusion characteristics (high blood volume fraction, rapid transit time, focal hyperpermeability and/or high fluorodeoxyglucose (FDG) metabolism) following chemotherapy correlates with a poor treatment response.

Due to the difficulties of relating clinical perfusion imaging with the underlying tumor structure and function, intravital microscopy (IVM) studies in live animals are becoming increasingly popular \[35, 36\]. Using video-rate laser-scanning microscopy, blood flow velocity, flux and hematocrit can be measured by tracking trajectories of fluorescent red blood cells (RBCs) \[37\]. Concomitant injection of a fluorescent tracer allows measurement of shear
rate [37], blood volume fraction [38] and tissue permeability [38–42]. These physiological parameters can be related to local variations in gene expression, enzyme activity, pH, metabolites and other parameters of interest (reviewed in [36]) by simultaneously imaging multiple fluorescent reporters. A major advantage of IVM is that tumor perfusion can be characterized on a vessel-by-vessel basis, potentially leading to insights into how local variations in perfusion can affect nanotherapeutics delivery and treatment response [43].

In this paper, we describe a theoretical framework for the automated evaluation of IVM perfusion curves in order to model the delivery of nanotherapeutics. Our hypothesis is that tumor-specific perfusion features may be used to model nanotherapeutics accumulation; thus, this framework aims to transcend the challenges posed by the typically abnormal tumor vasculature. Primary tumor fragments, collected from triple-negative breast cancer patients and grown as xenografts in mice, were injected with a bolus of a 40 kDa FITC–dextran tracer and monitored at 30 fps using IVM. The fluorescence intensity of each vessel was measured over time to yield a heterogeneous set of arterial and venous perfusion curves on a tumor-by-tumor basis. Two features were considered: the time to arterial peak and the venous delay, which acted as inputs for a fuzzy c-mean (FCM) supervised classifier. The data were classified into three defined groups (poorly vascularized, well vascularized and ‘in between’ vascularized), which were correlated to experimental nanoparticle accumulation measurements. We find that this approach enables an automated ranking of tumor vascular perfusion in order to model the delivery of nanotherapeutics. Using an independent validation set, we demonstrate that new samples can be mapped into the feature space to determine their perfusion ranking and hence estimate their nanoparticle retention. A major strength of this approach is that it enables the ranking of tumors and evaluation of their behavior in an automated manner without requiring PK models.

2. Materials and methods

2.1. Silicon particle fabrication

Plateloid nanoporous silicon particles of dimension 1000 × 400 nm were fabricated in the Microelectronics Research Center of The University of Texas at Austin [14]. Particles were fluorescently labeled for IVM as described in [14]. Particles were stored in IPA at 4 °C and then washed five times with phosphate-buffered saline immediately before use in vivo.

2.2. Animal models

BCM-2665, BCM-2147, BCM-4195 and BCM-3887 human cancer-in-mice xenografts were generated in SCID/beige mice (Harlan Laboratories, Wilmington, MA) by subcutaneous hetero-implantation of human tumor fragments in the mammary pad after gross removal of the epithelium [44]. These slow-growing tumor lines were selected for model calibration due to their highly reproducible vascularization from mouse to mouse. MDA-MB-231 triple-negative human breast cancer cells (ATCC) were grown orthotopically in nude mice as a validation set. Three days prior to imaging, mice received a one-time intravenous injection of DiD-labeled autologous RBCs (∼3% hematocrit) [14] for visualization of blood flow dynamics. When tumors reached 3–5 mm in diameter, mice were anesthetized with isoflurane and prepared for IVM using an aseptic skin-flap procedure [14]. Tumors were cover slipped using a custom coverslip mount for the duration of the experiment. 1–4 h after particle injection, mice were sacrificed by gravity perfusion with saline followed by 10% neutral buffered formalin.
All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at The Methodist Hospital Research Institute.

2.3. Intravital microscopy image acquisition

Live animals were imaged on an upright Nikon A1R laser scanning confocal microscope customized for IVM [43]. First-pass perfusion was imaged using a 4× magnification objective (3.2 × 3.2 mm) for 5 min at 30 frames per second (fps) immediately following an intravenous bolus of a 40 kDa FITC–dextran tracer (1 μM in 50 μl PBS; Invitrogen). High-resolution time-lapse images of particle accumulation were acquired across multiple fields-of-view (FOVs) for up to 4 h at 45–60 s intervals (7 fps) following a one-time intravenous injection of 5 × 10⁸ particles. A minimum of 20 randomly selected, non-overlapping 1000 × 500 μm FOVs were acquired in parallel for each animal. All image acquisitions were performed by simultaneous excitation of tissue with 488, 541 and 640 nm lasers. Emissions were collected by photomultiplier tubes with band-pass filters of ∼30–35 nm width centered at 525 nm (FITC–dextran tracer), 579 nm (silicon particles) and 670 nm (RBCs).

2.4. Quantification of particle accumulation

To determine particle accumulation as a function of time, the number of particles per FOV was quantified across each time-lapse video using image-processing techniques adapted from [14, 43]. The 12-bit images were split by color, the single pixel noise was removed using a minimum noise filter and individual particles were identified using a binary mask. Real-time particle accumulation curves (the number of particles mm⁻³) were produced by automated counting of the appropriate particles in each FOV, summing particle counts across all FOVs at each time point and then dividing by the sampled volume [14]. All IVM quantifications were performed using Nikon NIS Elements v4.0 software.

2.5. Quantification of tracer perfusion

IVM first-pass perfusion videos acquired at 30 fps were processed as follows: stills were extracted at ten-frame intervals and aligned relative to each other, yielding 3 fps motion-stabilized videos. Circular regions-of-interest (ROIs) were randomly placed inside vessel segments between branching points, ensuring that there was at least 1 pixel of space between ROIs and vessel margins. Multiple arterioles, venules and capillaries (∼10–200 μm diameter) representing the full spectrum of flow dynamics were selected for analysis, yielding approximately 30–60 ROIs per video. The mean fluorescence intensity of each ROI was measured and plotted as a function of time. Background fluorescence for intensity normalization was measured on a frame-by-frame basis using a non-perfused ROI. Measurement stability with regard to ROI placement was assessed by sampling sets of five identical ROIs within at least ten individual vessels per video. All IVM quantifications were performed using Nikon NIS Elements v4.0 software.

2.6. Perfusion classification

Tracer perfusion curves obtained for each tumor replicate were classified based on two features: the time to reach peak arterial perfusion and the delay between arterial and venous perfusion.
Arterial and venous signals were distinguished on a video-by-video basis by plotting peak vessel intensity versus vessel diameter, which was found to yield two distinct vessel groupings. Randomly selected ROIs were monitored visually to confirm that the vessels were correctly grouped. The time to peak arterial perfusion was determined by normalizing each arterial curve to obtain a distribution density function. The highest peak was assigned the highest probability of the signal and the time to reach it was termed the time to arterial peak. Since the signals had different durations, the time to arterial peak was normalized by dividing by the signal duration. The perfusion signals were thus represented as random variables with a number of values, each with a different probability. The set of possible values associated with each random variable was mathematically treated as a distribution. The venous delay, defined as the lag time between tracer appearance in the arterial system and the venous system, was similarly assessed by obtaining distribution density functions and normalizing by the signal duration. Due to the complexity of the vascular network, no attempts were made to visually pair specific arteries with veins; instead, the vascular system was treated as a closed-loop system with a single input and output.

The time to arterial peak and venous delay were measured for each tumor replicate, with the final value representing the expectation (\( E \)) of all the probabilities for each set of veins and arteries:

\[
E (\text{Feature 1}) = \sum_{i=1}^{n} P_{\text{vein}}(Y_i), \quad E (\text{Feature 2}) = \sum_{i=1}^{n} P_{\text{artery}}(X_i),
\]

where \( P_{\text{vein}}(Y_i) \) is the probability of the venous delay and \( P_{\text{artery}}(X_i) \) is the probability of the arterial peak.

2.7. Automated tumor ranking by fuzzy c-mean classification

Tumors were ranked according to degree of vascularization using an FCM classifier. Originally introduced by Bezdek [45], this technique ranks the data based on a membership grade assigned to each cluster. Two functions were utilized: a membership function (which establishes the degree of membership for points within a data cluster) and an objective function (which determines the position of the cluster center). Briefly, clustered data points were assigned an initial cluster center value. A membership function was then applied to determine the degree to which each given sample belongs to the cluster. The center of the clusters was changed iteratively to maximize the degree of belonging for all clustered data points. The unsupervised algorithm was stopped when the membership function became sufficiently small.

Tumors were ranked according to the time to arterial peak and venous delay by utilizing the objective function

\[
\min \left\{ J_m = \sum_{i=1}^{N} \sum_{j=1}^{c} u_{ij}^m \| x_i - c_j \|^2 \right\}, \quad 1 < m < \infty,
\]

where \( m \) is the degree of fuzzification, \( \| x_i - c_j \|^2 \) is the distance (or norm) that specifies the similarity between the data and the center of the clusters, \( c_j \) is a vector specifying the coordinates of the center of the clusters, \( x_i \) is the vector of the input data and \( u_{ij}^m \) is the membership degree of the \( i \)th input. Optimization of the objective function involved changing the exponential
membership function in which

\[ u_{ij} = \frac{1}{\sum_{k=1}^{c} \left( \frac{\|x_i - c_j\|}{\|x_i - c_k\|} \right)^{2/m}} \quad \text{and} \quad c_j = \frac{\sum_{i=1}^{N} u_{mij} \cdot x_i}{\sum_{i=1}^{N} u_{mij}}. \]

The condition \( \|U^k - U^{k-1}\| < \varepsilon \) was implemented as the algorithm stop function. All FCM classifications were performed in an automated manner using Matlab 7.10.0 (R2010a).

2.8. Model validation

Model validation was performed in a blinded manner. Individual MDA-MB-231 tumors were classified based on arterial peak and venous delay measurements and then ranked by membership to the closest cluster center. Estimates of particle accumulation were generated by plotting tumor ranks on a curve calibrated with the training set (BCM-2665, BCM-2147, BCM-4195 and BCM-3887 tumors).

3. Results

3.1. Nanotherapeutics delivery is heterogeneous from tumor to tumor

Real-time IVM of silicon particle accumulation in tumors revealed tumor-specific differences in particle accumulation. Particle accumulation was quantified across triple-negative breast tumor biopsies, which were collected from four different patients (BCM-2147, BCM-2665, BCM-3887 and BCM-4195) and grown as xenografts in immunocompromised SCID/Beige mice. Figure 1 shows sample images and kinetic data for the accumulation of 1000 × 400 nm particles in the vasculature of the 2147 and 4195 tumors. Mice treated with identical numbers of particles (5 × 10^8) showed distinct differences in the number of particles accumulated. Particle accumulation varied between tumors derived from different patients, as well as within tumor replicates. The 2147 mouse shown in figure 1, for example, accumulated particles at a density of \( \sim 27900 \) particles mm\(^{-3}\), whereas the 2147 replicates as a whole (\( n = 3 \)) accumulated particles at a density of \( 27500 \pm 1450 \) particles mm\(^{-3}\). Similarly, the 4195 mouse shown in figure 1 accumulated particles at a density of \( \sim 2920 \) particles mm\(^{-3}\), whereas 4195 replicates (\( n = 4 \)) accumulated particles at a density of \( 3450 \pm 400 \) particles mm\(^{-3}\). The largest difference in particle accumulation was observed between tumors derived from different patients, leading us to speculate that tumor-specific differences in perfusion play an important role in nanoparticle delivery.

3.2. Quantification of tracer first-pass perfusion

The first-pass perfusion of an intravenous bolus of a 40 kDa FITC–dextran tracer was recorded in real time (30 fps) using IVM. Representative IVM videos of the 2147 (video 1) and 4195 (video 2) tumors perfusion can be found in the online supplementary data (available from stacks.iop.org/NJP/15/055004/mmedia). Frame-by-frame analysis of tracer intensity in selected ROIs yielded perfusion time curves for individual vessels. A representative video of the frame-by-frame analysis (video 3) can be found in the online supplementary data. Figure 2 highlights sample arterial and venous curves generated by the 2147 and 4195 tumors implanted into mice. Several key features can be observed: The arterial curves (red) are generally characterized
Figure 1. Accumulation of 1000 × 400 nm silicon particles in the vasculature of human breast cancer xenografts. (a) Representative IVM images of silicon particle (red) adhesion to the walls of perfused blood vessels, collected 1 h after particle injection. Vessel walls were delineated via a 40 kDa FITC–dextran tracer (green) and vessel perfusion was identified by the presence of moving RBCs (blue). Inset: a magnified view demonstrating that individual particles can be readily distinguished for quantification purposes. Scale bar = 50 µm. (b) Representative particle accumulation kinetics as measured by IVM, shown for individual 2147 and 4195 mice. Differential particle accumulation is observed, with the 2147 tumors showing more rapid particle accumulation and a higher quantity of adherent particles when compared to 4195 tumors.

by a rapid increase in fluorescence intensity which plateaus within the first minute, drops off and levels out. The venous curves (blue) demonstrate a more gradual increase in fluorescence intensity, resulting in a delayed plateau. A large number of such curves (∼30–60) were generated for each implant, allowing characterization of intratumoral heterogeneity. Differences were observed in peak intensity, time to arterial peak and venous delay within each tumor, suggesting that vascularization can be heterogeneous within a given tumor. Independent stability analyses were performed for each video to confirm that ROI placement did not have a significant impact on the rate of tracer influx and that the variations in signal intensity at any given time point fell within the overall signal noise. Data were considered robust and included for classification when these conditions were met.

3.3. Identification of tissue classifiers

Heterogeneities in intratumoral perfusion make it difficult to apply standard curve-fitting models for perfusion classification. The first-pass perfusion signal in tumor arteries, for example, does not necessarily rise very quickly to a maximum as would be expected in normal tissue. This was particularly evident for the 4195 and 3887 biopsy implants, in which a slow rise in arterial fluorescence was followed by little or no decrease, indicative that the tracer was already fully...
mixed in the blood by the time of arrival. In contrast, the 2147 and 2665 tumors behaved more like normal arteries in that their fluorescence intensity rapidly peaked and then declined immediately after the peak. These observations suggest that the time it takes for arterial flow to reach its peak is a critical feature of tumor perfusion. Accordingly, we generated a distribution density function in which the highest peak has the highest probability of signal, and the time to reach the peak following tracer entry was defined as the ‘time to arterial peak’.

The perfusion of the tumor venous system showed distinct abnormalities as well. In normal tissue, the duration from the time blood enters an artery until it reaches the corresponding vein is a few seconds. In the tumors in this study, however, this process varied from a few seconds to over a minute. The 2147 tumor in figure 2, for example, showed a delay of ~30–90 s between when the tracer appeared in the arteries and when it appeared in the veins, whereas the 4195 tumor showed a venous delay of ~10–30 s. Thus, we postulated that venous delay is also an important feature of tumor perfusion. Accordingly, we applied statistical rules to measure the probability of this delay.

3.4. Classification of tumor vascularity

The time to arterial peak and venous delay was calculated for each biopsy implant and mapped to a single point within a two-dimensional feature space. The final value of a given feature is

Figure 2. Quantification of tumor first-pass perfusion following injection of a 40 kDa FITC–dextran tracer. (a) Representative ROIs selected for time measurements of fluorescence intensity. Circular ROIs were randomly defined inside arterioles, venules and capillaries, between branching points, yielding approximately 30–60 ROIs per video. (b) Representative arterial (red) and venous (blue) perfusion-time curves of individual vessels, measured for single 2147 and 4195 tumors. Variations in the time to arterial peak and in venous delay are observed within each tumor. The videos from which the data have been derived can be found in the online supplementary data (available from stacks.iop.org/NJP/15/055004/mmedia).
Table 1. The calculated expectation of individual tumor features.

| Tumor type | Feature 1: venous delay | Feature 2: arterial peak |
|------------|--------------------------|--------------------------|
| 1          | 2147                     | 0.0415                   |
| 2          | 2147                     | 0.1608                   |
| 3          | 2147                     | 0.1468                   |
| 4          | 2665                     | 0.0300                   |
| 5          | 3887                     | 0.0187                   |
| 6          | 3887                     | 0.0961                   |
| 7          | 3887                     | 0.2766                   |
| 8          | 3887                     | 0.0540                   |
| 9          | 4195                     | 0.0830                   |
| 10         | 4195                     | 0.1822                   |
| 11         | 4195                     | 0.1334                   |
| 12         | 4195                     | 0.3526                   |

Figure 3. Application of the FCM classifier partitions the data set into three distinct categories. The numbers denote the individual tumor replicates as listed in table 1. Three classes are marked and the samples are shown with the same color as the center of the class. The red crosses represent poorly vascularized cases, the blue triangles belong to the well-vascularized class and the green circles denote the ‘in between’ cases.

The data were separated into three different classes based on the pattern of scatter: the first includes cases that are poorly vascularized (i.e. short time to peak and short venous

the expectation of all the probabilities of the vessels imaged over a 10 mm² FOV. Table 1 shows the results of the two features calculated for each implant. When plotted in a two-dimensional feature space, the complexity of the data becomes apparent (figure 3). The scattering of these points reflects both intra- and inter-tumoral heterogeneities. If no intra-tumoral heterogeneities were present, for example, we would expect to see four tight clusters of data, one for each set of tumor replicates. Since the data do not fall in a single line, a linear classification is not possible.

New Journal of Physics 15 (2013) 055004 (http://www.njp.org/)
delay), the second includes cases that are well vascularized (i.e. long time to peak and long venous delay) and the third class is neither poorly nor well vascularized (‘in between’ vascularized). These ‘in between’ cases can represent tumors of homogeneous vasculature with intermediate perfusion properties or tumors of heterogeneous vasculature with regions with differing perfusion properties. No restrictions were placed on the classification of very well or very poorly vascularized cases. Figure 3 shows the data set in the two-dimensional feature space after applying the FCM classifier.

The data were ranked by taking into account both the distance of each data point to the center of its cluster and a weighted term for each of the measured features:

$$\text{rank}(p) = w_1 \times \text{feature 1 (venous delay)} + w_2 \times \text{feature 2 (arterial peak)},$$

where $p$ is the tumor replicate in each class and $w_1$ and $w_2$ are the weighted terms for the venous delay and arterial peak, respectively. We note that there exist numerous optimization algorithms to determine such weighted terms [46, 47]. Here, the values for $w_1$ and $w_2$ were chosen by separately calculating the first moment of each feature in each class. Since each feature is described as a separate distribution function, $w_1$ and $w_2$ were selected so that $w_1 + w_2 = 1$. The other constraint, which is based on the observation that normal vasculature is usually associated with a higher probability of venous delay than rapidly perfused tumors, assigns a higher weight to $w_1$. The ratio $r$ of the mean of the two features in each class thus defines the second constraint as $(w_1/w_2 = r)$. Using our experimental data, we calculate $w_1 = 0.6$ and $w_2 = 0.4$. Note that we do not choose the Euclidean distance from the samples to the center of the classes as the ranking criteria since the accuracy of such an approach is proportional to the sample number. Figure 4 shows the automated tumor ranking from poorly vascularized tumors (left) to well-vascularized tumors (right). Despite the small sample size, it can be observed that there is a large dynamic range ($\sim 9.5$-fold) in the measured values. Interestingly, replicates from the same patient (No. 1–3 (2147), No. 5–8 (3887) and No. 9–12 (4195)) do not group together, which reflects intra-tumoral heterogeneities in growth and vascularization.

**Figure 4.** Automated ranking of the implants based on weighted feature probabilities.
Table 2. Comparison between classification, automated ranking and experimentally derived nanoparticle accumulation measurements. Mean values for each tumor type are given, followed by the individual measurements. Mean values are presented as ±1 standard deviation. Tumor vascularity is indicated by − (poorly vascularized), ± (in between) or + (well vascularized).

| Patient | Vascularity classification | Automated rank value | Particle accumulation (number of particles mm⁻³) |
|---------|---------------------------|----------------------|-----------------------------------------------|
| 1       | 2147                      | − 0.1160 ± 0.0661    | 27 500 ± 1450 (28 690)                       |
| 2       | 2147                      | ± (0.1534)           | (25 880)                                      |
| 3       | 2147                      | ± (0.1550)           | (27 930)                                      |
| 4       | 2665                      | − 0.0293             | 30 620 (30 620)                               |
| 5       | 3887                      | − 0.1434 ± 0.1116    | 10 810 ± 5400 (8080)                         |
| 6       | 3887                      | ± (0.1560)           | (18 800)                                      |
| 7       | 3887                      | + (0.2959)           | (9410)                                        |
| 8       | 3887                      | − (0.0722)           | (6960)                                        |
| 9       | 4195                      | − 0.1714 ± 0.1157    | 3450 ± 400 (3410)                            |
| 10      | 4195                      | ± (0.1617)           | (3620)                                        |
| 11      | 4195                      | − (0.1103)           | (2920)                                        |
| 12      | 4195                      | + (0.3369)           | (3850)                                        |

Particle accumulation was measured for all tumor replicates evaluated here. Table 2 shows a comparison between tumor classification, automated ranking and particle accumulation on a tumor-by-tumor basis. Since the classification and ranking schemes do not show a consistent ranking, we cannot compare them directly with the experimental results. We therefore calculated the average value of these features for each patient. Comparison of the averaged patient ranking with the averaged experimental measurement suggests that tumors classified as ‘poorly vascularized’ would uptake the highest number of circulating particles, whereas tumors classified as ‘well vascularized’ would uptake the lowest number of particles. This trend is shown in figure 5, where the automated ranking appears inversely proportional to particle accumulation. Data were fit to a second-order polynomial based on the observation that 1000 × 400 nm plateloid particle accumulation is constrained by tumor-specific physiological transport phenomena [48]. The 2665 tumor, although a single replicate, had relatively little impact on the classification scheme and was therefore considered robust and included in the fit. Thus, the relationship between tumor rank and particle accumulation appears to be nonlinear, with small changes in the upper ranks yielding large changes in particle accumulation.

Triple-negative MDA-MB-231 xenografts were generated for model validation. Grown simultaneously in littermates for 30 days under identical conditions, these tumors nevertheless demonstrated significant differences in tumor vascularization, particle accumulation and ranking. Figure 6(a) highlights the morphological differences observed under brightfield illumination and following FITC–dextran injection. Cumulative particle accumulation, as measured by IVM, was found to vary by as much as ∼30-fold across the five tumors studied (figure 6(b)). These tumors were individually classified and ranked in a blinded manner (table 3).

The calculated tumor ranks were found to range from 0.035 (tumor 4, poorly vascularized) to 0.378 (tumor 1, well vascularized). Figure 6(c) shows the predicted and measured particle
Figure 5. Comparison of tumor rank to experimentally observed particle accumulation. (a) Average tumor rank, grouped by patient number. Note that sample 2665 is a single replicate. (b) Average particle accumulation, grouped by patient. Particle accumulation is observed to be inversely proportional to tumor rank. (c) Plot of the nonlinear relationship between the average particle accumulation and the average tumor rank.

accumulation values, plotted by tumor rank. The three tumors predicted to show high particle accumulation (> 20000 particles mm$^{-3}$) correlated in a statistically significant manner (two-tailed test with $\alpha = 0.05$) with the model prediction ($R = 0.99$ as measured by Pearson product moment correlation), while those with relatively high ranks (>0.18) showed low particle accumulation as expected.

4. Discussion

The collection and interpretation of perfusion data present significant challenges at both the clinical and the preclinical level. In a clinical setting, it is difficult to acquire morphological and perfusion information simultaneously following a single dose of contrast agent; thus most researchers have to choose between studying dynamic or architectural features [19]. While early evidence suggests that a careful combination of both data sets may ultimately improve the accuracy of breast cancer imaging [49, 50], researchers are yet to identify perfusion features that may have diagnostic or prognostic value. For this reason, we utilized IVM, a preclinical imaging modality with high spatial ($\leq 2 \mu m$) and high temporal ($\leq 30$ fps) resolution that is uniquely suited for studying tumor pathophysiology in animal models [36]. Collection of data at these scales allows us to detect heterogeneities in tumor vascular structure and function, but at the same time introduces new complexities into the data analysis.

Here we utilized IVM to simultaneously collect both perfusion and particle accumulation kinetics in patient-derived xenografts. Vessel perfusion was measured on a vessel-by-vessel
Figure 6. Vascularization, particle accumulation and ranking of MDA-MB-231 xenografts. (a) Brightfield and fluorescence microscopy images of five individual tumors grown under identical conditions (top to bottom: rows 1–5). Significant differences in vascular morphology were observed, as well as local differences in vessel permeability resulting in tracer extravasation. Scale bar = 200 µm. (b) Cumulative particle accumulation, as measured by IVM, ranged widely from ∼1000 to 34 000 particles mm⁻³. (c) Predicted (dashed line) and measured (blue solid square) particle accumulation values, plotted by tumor rank. The degree of particle accumulation was categorized by position along the predicted accumulation curve (from figure 5(c)). High accumulation: >20 000 particles mm⁻³; moderate accumulation: 5000–20 000; low accumulation: <5000 particles mm⁻³.

basis, in order to yield a large set of arterial and venous perfusion curves whose variability reflects local differences in vessel structure and function. Features of interest were directly extracted from the measured data sets by using an FCM supervised classifier. Since our goal was to model nanoparticle accumulation based on tumor perfusion characteristics that vary with location and time, we chose not to extrapolate global tumor parameters such as the mean transit time or blood volume (see the appendix). While such measurements might serve as acceptable classifiers, interpolation of each data set into a single-intensity curve would effectively discard all measurements of intratumoral heterogeneity.
Table 3. Automated classification and ranking of MDA-MB-231 tumors. Tumor vascularity is indicated by − (poorly vascularized), ± (in between) or + (well vascularized).

| Vascularity classification | Automated rank value | Particle accumulation (number of particles mm$^{-3}$) |
|---------------------------|----------------------|--------------------------------------------------|
| 1                         | +                    | 0.3779                                           |
| 2                         | −                    | 0.0977                                           |
| 3                         | −                    | 0.0920                                           |
| 4                         | −                    | 0.0352                                           |
| 5                         | ±                    | 0.2245                                           |
|                           |                      | Predicted                                        |
|                           |                      | 0                                                 |
|                           |                      | 28 940                                           |
|                           |                      | 29 960                                           |
|                           |                      | 31 610                                           |
|                           |                      | 1900                                             |
|                           |                      | Measured                                         |
|                           |                      | 1070                                              |
|                           |                      | 28 310                                           |
|                           |                      | 34 300                                           |
|                           |                      | 26 070                                           |
|                           |                      | 1900                                             |

To analyze our complex sets of perfusion measurements, we developed a framework to automatically perform tumor classification and ranking without relying on PK models. Traditional dynamic imaging techniques require PK models to analyze the time dependence of contrast signals in order to extrapolate features such as the tumor blood volume, blood flow, permeability and the size of the extravascular space. The accuracy of such models is ultimately limited by the underlying complexity of the tumor pathophysiology and our ability to model these complexities [51]. For this reason, we chose to rank tumors based on direct measurements of the raw data, without using PK models as an intermediary. Although our data sets failed to yield a simple relationship between the shape of the perfusion curves and tumors from specific patients, we were able to extract the time to arterial peak and venous delay as discriminating features for tumor classification and ranking. By measuring these two features from experimental IVM data sets, we showed that individual tumors could be automatically ranked according to tumor perfusion. Calibration of the model with IVM measurements of particle accumulation allowed estimation of tumor behavior without requiring any underlying assumptions about tissue structure or function, or heterogeneities contained therein.

The calculated ranks are a direct reflection of tumor heterogeneity, since the classified features incorporate the probabilities collected over a large number of vessels. If no intratumoral heterogeneities were present, for example, we would expect replicates derived from the same patient to yield identical results. Instead, differences in both tumor perfusion and particle accumulation are observed from patient to patient, as well as within tumor replicates. When tumor ranks are averaged by patient, it appears that particle accumulation is preferentially associated with poorly vascularized tumors (e.g. short venous delay and short time to arterial peak). This relationship, plotted in figure 5(c), is dependent on tumor heterogeneity. As tumor perfusion becomes more heterogeneous (with respect to the venous delay or time to peak), the tumor rank will shift along the accumulation curve. Thus, we expect changes in tumor perfusion to yield differences in nanotherapeutics delivery.

The xenograft tumor sets studied here highlight the importance of selecting appropriate animal models for the study of biophysical transport phenomena. Xenografts grown from human cell lines are generally fast growing and show large variations in vascularization from mouse to mouse. As a result, the statistical power animal studies is largely dependent on the scope, quality and amount of samples analyzed. Our findings suggest that tumors implanted...
as tissue fragments, while generally slower growing (~2–4 months), show a higher degree of reproducibility when evaluated in terms of tissue perfusion and particle accumulation. This allowed us to calibrate the model with a small set of tumors. In contrast, tumors derived from MDA-MB-231 cells varied much more widely than the tumor lines, translating into a ~10-fold range in ranking. Predictions of particle accumulation in individual tumors matched well with experimentally measured values, supporting our hypothesis that tumor-specific perfusion features may be used to model nanotherapeutics accumulation.

This observation has interesting implications for patient therapy. Successful nanotherapy relies largely on the number of nanoparticles that are retained by tumor tissue [43]. Patients treated with anti-angiogenic agents, for example, may accumulate different numbers of nanotherapeutics and thus respond very differently after vascular remodeling. Vascular normalization via VEGF targeting has been reported to elevate blood perfusion [52] and modulate nanotherapeutics size selectivity [53]. It is not yet known, however, how decreases in vessel leakiness may compromise the advantages gained from increased convection. Based on our model, we would expect vascular normalization to improve particle delivery by simultaneously increasing perfusion and decreasing intratumoral heterogeneity. Such a hypothesis could be readily tested in animals using the methodologies outlined here.

From a clinical perspective, there is great interest in identifying tumor-specific perfusion parameters that govern nanotherapeutics delivery. Patient eligibility for nanotherapeutics is generally indicated after failure of prior chemotherapy. The IVM data presented here suggest that select groups of patients may highly benefit from nanotherapy while others may not. Based on our initial findings, we propose three ranges of particle accumulation: high (> 20 000 particles mm\(^{-3}\)), moderate (5000–20 000) and low (<5000 particles mm\(^{-3}\)). We previously calculated that therapeutic silicon particles retained at a density of > 15 000 particles mm\(^{-3}\) are likely to deliver sufficient drug for achieving therapeutic benefit at subclinical doses [43]. Thus, tumors whose rankings yield moderate to high accumulation are expected to benefit the most from the use of nanotherapeutics over conventional chemotherapy. The approach described here, which combines the high spatial and temporal resolution of IVM with mathematical modeling, is a powerful tool for studying this issue in pre-clinical models. Both perfusion and particle accumulation, for example, can be studied in the same animal on a vessel-by-vessel basis in order to identify the mechanisms driving particle delivery and accumulation. Since we expect nanoparticle delivery to vary based on physical properties such as particle size, shape and surface chemistry [14], a large variety of different nanoparticles may be imaged via IVM and utilized to create a library of perfusion model calibrations. While IVM itself is not suitable for use in patients, the ultimate goal of such studies would be to identify specific parameters that can be measured using existing clinical instruments for personalized selection of appropriate nanotherapeutics.

Although these analyses were performed with only a small set of tumors, they serve to highlight the importance of tumor ranking. The methodology we describe here allows an unbiased, automated comparison between different tumors. New samples can be mapped directly into the feature space in order to determine their perfusion ranking. The number of classes and their center positions are readily adapted using this learning methodology so that the model becomes further refined as it receives more inputs. Each input results in a discrete rank value that can be used to quantitatively compare the pathophysiology of one tumor against other measured tumors. Such rankings can be correlated with a variety of quantifiable physiological parameters in order to evaluate the behavior of a given tumor.
The same methodology could be applied to a variety of different cancer types to generate tumor-specific information. We expect this to be particularly useful for glioblastoma, where perfusion parameters are thought to correlate with tumor grade, aggressiveness and prognosis [54–57]. Furthermore, estimates of particle accumulation based on perfusion characteristics could be coupled with advances in multiscale models of tumor vasculature and blood flow [58] in order to predict response to treatment. In our recent works, we have simulated the effect of tumor vascularization and blood flow on particle transport and accumulation within solid tumors [43, 59]. Theoretical calculations of arterial peak and venous delay, generated via such models, could be automatically evaluated using the framework proposed herein, allowing improved estimation of particle accumulation in combination with patient-specific measurable characteristics such as vascular density and tumor size [59].

We note that additional features can be extracted from the perfusion curves beyond the two described here, such as single-vessel flow rate and tracer/particle throughput, in order to improve model sensitivity and specificity. In this pilot study, we describe the simplest automated method for estimating particle delivery based on readily identifiable tumor perfusion characteristics. Clustering of our data sets shows that the relationship between tumor rank and particle accumulation is nonlinear and that tumor rank is not always reproducible across tumor replicates. A single, general deterministic definition for particle accumulation is unlikely to found, and as such, makes it difficult to assign a sensitivity and specificity as is common with clustering techniques. Nevertheless, our initial implementation enabled us to determine that there is an inverse relationship between particle accumulation number and vascularization rank, and that this holds true for the test data set. While it is still unclear at this time how many features are needed for model improvement, increasing the sample size and feature number is expected to greatly improve the statistical significance of such studies.

In summary, here we present an automated technique for the theoretical modeling of nanotherapeutics delivery based on heterogeneous sets of whole arterial and venous perfusion curves collected at the single vessel level. Unlike conventional perfusion studies, in which tumor tissue and its vasculature are treated as individual homogeneous compartments for the extrapolation of PK parameters, tumors were classified and ranked without requiring any underlying assumptions about tissue structure or function, or heterogeneities contained within. The time to arterial peak and venous delay, measured directly from the experimental data, proved sufficient for discriminating between individual tumors. The resulting tumor rankings correlated inversely with experimental nanoparticle accumulation measurements, allowing the estimation of nanotherapeutics delivery. With proper calibration, these methodologies may enable the study of a variety of nanoparticles in different tumor models.

Acknowledgments

This work was supported by an NIH/NCI Physical Sciences—Oncology Center (PS-OC) Young Investigator Trans-Network Award to AV and HF as a part of the Methodist (U54CA143837) and USC (U54CA143907) PS-OC Centers. LB, ML and JC acknowledge funding from the NIH (R01CA138197 and U54CA149196-01) and the Breast Cancer Research Foundation. MF acknowledges funding from NIH/NCI PS-OC grant number U54CA143837 and additional research support from DoD/BCRP (W81XWH-09-1-0212), NIH/NCI (U54CA151668) and Ernest Cockrell Jr Distinguished Endowed Chair.
Appendix

Blood flow is commonly modeled by treating arteries as conduits that supply oxygenated blood and veins as conduits that carry away deoxygenated blood. Oxygen and other nutrients are exchanged with tissues at the level of the capillary beds, which are situated between the arteries and veins. Contrast agents injected into the bloodstream circulate through this closed-loop system and can be visualized using a variety of imaging modalities. Contrast agents are generally assumed to remain confined within the vasculature. This is not necessarily true, however, for fenestrated tumors, hemorrhages, strokes and other pathological conditions, which can introduce significant challenges to the measurement of perfusion curve features [23–27].

The main idea underlying the analysis of perfusion curves is to quantitatively measure the amount of blood flowing through a tissue. The minimum information needed is the concentrated intensity signal of arteries, the concentrated intensity signal of veins and the spatial volume under consideration. If we consider an inlet of one artery and an outlet of one vein, we can calculate the blood volume based on conservation of mass:

\[
m_{\text{tissue}}(t) = m_{\text{in-artery}}(t) - m_{\text{out-vein}}(t).
\]

Methods for analyzing perfusion curves can be classified into two general categories: deconvolution based and direct measurement (non-deconvolution) based [23, 60]. The first category involves deconvolving the unknown tissue impulse from measurements collected during the first pass of a contrast agent through the tumor and a reference artery. Non-deconvolution techniques extract parameters directly from the whole tissue perfusion curve, which is collected following multiple passes of a contrast agent [23, 25, 47]. There is currently no technical agreement on when the deconvolution-based methods are preferable to direct measurements [60].

To determine blood flow using deconvolution-based methods, the contrast agent signal intensity in the vein is theoretically defined as the convolution of the contrast agent signal intensity of the artery with \( h(t) \), which is the probability density function of the transit time \( t \) of the blood volume through the capillary bed. The contrast signal intensity of the vein is then computed as follows:

\[
c_{\text{vein}}(t) = \int_{-\infty}^{+\infty} c_{\text{artery}}(\tau) h(t - \tau) \, d\tau.
\]

The mean transit time is the first moment of the probability density function \( h(t) \):

\[
\text{MTT} = \int_{0}^{\infty} t h(t) \, dt.
\]

The function \( h(t) \) typically follows a gamma distribution. We note that currently research activity is in progress with the aim to fit the gamma variate function model and extract its parameters in order to calculate the features of blood perfusion curves [61, 62].

The concentration signal intensity in the artery \( c_{\text{artery}} \) is the input to the tissue. The tissue response \( c_{\text{tissue}} \) can then be determined through the convolution of the artery signal and \( r(t) \), which is the residue function \( r(t) = 1 - \int_{0}^{t} h(t) \, dt \):

\[
c_{\text{tissue}} = \text{Blood volume} \int_{-\infty}^{+\infty} c_{\text{artery}}(\tau) r(t - \tau) \, d\tau.
\]

Applying deconvolution, this technique enables determining \( r(t) \) with the known tissue concentration signal intensity and artery concentration signal intensity. The method provides an
accurate solution to find \( h(t) \), which enables finding the volume of blood in the tissue, and hence the flow according to

\[
\text{Blood flow} = \frac{\text{Blood volume}}{\text{Mean transit time}}.
\]

Using non-deconvolution-based methods, the blood volume can be calculated directly from the experimentally obtained perfusion curves as follows:

\[
\text{Blood volume} = \max \left( \frac{d\text{tissue}(t)}{dt} \right) / \max(\text{c}_{\text{artery}}(t)).
\]

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