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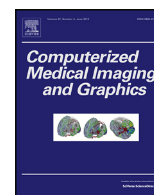

Research paper

Improved threshold selection for the determination of volume of distribution of nanoparticles administered by convection-enhanced delivery

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ABSTRACT

Nanotechnology, in conjunction with convection-enhanced delivery (CED), has gained traction as a promising method to treat many debilitating neurological diseases, including gliomas. One of the key parameters to evaluate the effectiveness of delivery is the volume of distribution (V_d) of nanoparticles within the brain parenchyma. Measurements of V_d are commonly made using fluorescent reporter systems. However, reported analyses lack accurate and robust methods for determining V_d. Current methods face the problems of varying background intensities between images, high intensity aggregates that can shift intensity distributions, and faint residual backgrounds that can occur as artifacts of fluorescent imaging. These problems can cause inaccurate results to be reported when a percentage of the maximum intensity is set as the threshold value. Here we show an implementation of Otsu's method more reliably selects accurate threshold values than the fixed-threshold method. We also introduce a goodness of fit value η that quantifies the appropriateness of using Otsu's method to calculate V_d. Adoption of Otsu's method and reporting of η may help standardize fluorescent image analysis of nanoparticles administered by convection-enhanced delivery.

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1. Introduction

Glioblastoma (GBM) is the most common and aggressive type of brain tumor in adults, with an annual incidence of 3.19 per 100,000 people (Mrugala and Chamberlain, 2008). Highly heterogeneous and invasive, GBM constitutes the most severe grade of malignant glioma and is associated with extremely poor prognoses. Despite multimodal therapy consisting of surgery, radiation, and chemotherapy, GBM patients have a median survival of only 15 months (Eramo et al., 2006). Primary brain tumors usually recur—after therapy—within 2 cm of the original site (Allard et al., 2009).

Treating GBM with chemotherapeutic agents poses major drug delivery challenges. First, most chemotherapeutic agents have short half-lives in blood circulation and the tumor microenvironment, and are easily metabolized or eliminated before eliciting their therapeutic effect. To tackle this challenge, polymeric nanoparticles have been used to protect fragile molecules from metabolism, offering the possibility of sustained release. Second, the blood brain barrier (BBB) is the primary interface between the blood and the brain interstitial fluid (ISF), and prevents 98% of small molecules and effectively 100% of large molecules from reaching the brain parenchyma when delivered by systemic administration (Pardridge, 2005).

The BBB can be bypassed using local delivery. Polymeric wafers implanted directly in the tumor cavity, such as Gliadel® (Fleming and Saltzman, 2002), represent one such method for direct delivery of chemotherapeutic agents. In clinical practice, implantation of Gliadel typically follows neurosurgical resection. However, this approach allows for only modest therapeutic improvement, likely due to its reliance on diffusion for drug penetration in the brain tissue (Fung et al., 1998). Indeed, drugs loaded in Gliadel® wafers achieved depth of penetration in tissue of about 1 mm (Hochberg and Pruitt, 1980), whereas GBM cells have been detected in areas remote from the primary lesion, including the corpus callosum (Kallenberg et al., 2013).

Convection-enhanced delivery (CED) constitutes another method for direct, local delivery, and allows for overcoming issues associated with therapeutic distribution. During CED, drugs are infused continuously in the brain tissue through a catheter connected to an infusion pump. Unlike polymeric wafers,

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CED establishes a pressure gradient, allowing for distribution of drugs over large volumes. Morrison et al. predicted that CED can increase the volume of distribution (V_d) of macromolecules by five-fold over simple diffusion (Morrison et al., 1994). Recent clinical trials showed that CED is safe and feasible; however, CED has not yet translated to improvements in clinical outcomes. In particular, it has been suggested that suboptimal V_d compromise the therapeutic efficacy of CED (Sampson et al., 2010). Numerous pre-clinical studies from several laboratories have combined the use of nanoparticles with CED, showing the importance of size, surface charge, and stability to ensure wide distribution of particles throughout the brain tissue. However, values of V_d vary greatly among these reports, and there is a lack of a standardized method to compare brain penetration of particle formulations after CED.

To evaluate the distribution of nanoparticles in the brain, the system is usually labeled using a fluorescent dye. Thin slices of brain tissue are then visualized using fluorescence microscopy, and these images are saved digitally for further analysis. It is assumed that fluorescence distribution reflects nanoparticle and drug penetration. Image processing requires extracting objects from their background. Thresholding involves separating each pixel into one of two classes, background or foreground, according to a calculated threshold value. Background refers to areas of the image with intensity values less than the specified threshold, and foreground refers to areas with intensity values greater than the threshold. In the ideal case, an image would have an intensity histogram characterized by a sharp valley between two peaks, each one corresponding to the foreground and background. Thus, the choice of a threshold is conceptually simple but often practically difficult. For example, a histogram with a flat and broad valley or peaks of unequal heights offers no easily discernable threshold (Yan, 1996).

Currently, most laboratories reported automated methods wherein a fixed threshold is set as a proportion of the maximal intensity of any particular pixel in the image (fixed-threshold method). However, this strategy has resulted in selections of thresholds that appear to be incorrect upon manual inspection, highlighting the need for a more reliable approach for image thresholding. Chow and Kaneko previously developed a method of automatic boundary detection for identifying the left ventricle in cineangiograms. Their technique involves approximating the histogram using a method of least squares. However, the assumption of Gaussian distributions often does not correspond well to real images, and thresholds are set according to local, rather than global, characteristics of the image (Chow and Kaneko, 1972). Weszka et al. previously developed a technique wherein the threshold is selected based on the Laplacian of the histogram. The choice of threshold corresponds to the region of maximal difference. However, one drawback of this approach is that it does not offer a measure of the "goodness" of a threshold selection (Weszka et al., 1974). Otsu's method is a nonparametric and unsupervised method of thresholding. This method distinguishes between foreground and background by minimizing the weighted within-class variance of foreground and background pixels, which is equivalent to maximizing the between-class variance (Otsu, 1979). Application of Otsu's method resulted in adequate separability as compared to other thresholding methods when applied to images of breast tumor cells (Jeong et al., 2005).

In this paper, we developed a MATLAB algorithm based on Otsu's method to analyze fluorescence images of nanoparticle distribution after CED. Upon visual inspection, this algorithm appeared more effective at image thresholding than the fixed-threshold method currently reported in the literature. This was observed when analyzing images of different particle types and encapsulated dyes, showing the universality of the method. Moreover, our algorithm also provided a parameter reflecting the "goodness" of the threshold that quantifies the appropriateness of the threshold selection, and can be used as a comparison criterion between studies.

2. Methods

2.1. Preparation of NPs

PEGylated SQ-Gem NPs loaded with BODIPY-CE dye (1% w/w) at a final concentration of 10 mg/mL in SQ-Gem were prepared by the nanoprecipitation technique as previously described (Couvreur et al., 2006). PLA-HPG NPs loaded with the DiA dye (0.2% w/w) at a final concentration of 100 mg/mL in PLA-HPG were prepared by the emulsion-evaporation technique as previously described (Song et al., 2017). Brain penetrating PLGA NPs loaded with Nile Red (0.2% w/w) at a final concentration of 100 mg/mL in PLGA were prepared by the emulsion-evaporation technique followed by centrifugation steps to recover small particles, as previously described (Saucier-Sawyer et al., 2016). TAMRA-PNA PLA-HPG particles were prepared by nanoprecipitation as follows. TAMRA tagged-PNA was suspended in water at a concentration of 1 mg/mL. The solution was emulsified into a mixture of DMSO and ethyl acetate (1:3 v/v) with 6 mg of PLA-HPG dissolved and sonicated to create a first emulsion. This final emulsion was then added dropwise to 6 mL of water (1:5 v/v ratio).

2.2. CED of NPs

Male Fischer 344 rats (6–8 weeks old, Charles River Laboratories) or C57BL6 mice (6–8 weeks old, Charles River Laboratories) were used. All procedures were performed in accordance with the guidelines and policies of the Yale Animal Resource Center and approved by the Institutional Animal Care and Use Committee. Surgical procedures were performed using standard sterile surgical techniques. Animals were anesthetized using a mixture of ketamine (75 mg/kg) and xylazine (5 mg/kg), as previously described. Animal's head was shaven, and the animal was then placed in a stereotaxic frame. After sterilization of the scalp with alcohol and betadine, a midline scalp incision was made to expose the coronal and sagittal sutures, and a burr hole was drilled above the striatum region (3 mm lateral to the sagittal suture and 0.5 mm anterior to the bregma for rat, 2 mm lateral to the sagittal suture from the bregma for mice). A 50 μL Hamilton syringe with a polyamide-tipped tubing, loaded with the NPs, was inserted into the burr hole to reach the striatum (depth of 5 mm from the surface of the brain for rats, depth of 3 mm from the surface of the brain for mice), and left to equilibrate for 7 min before infusion. A micro-infusion pump (World Precision Instruments, Sarasota, FL, USA) was used to infused 20 μL (rats) or 5 μL (mice) of NPs at a rate of 0.667 μL/min. Once the infusion was finished, the syringe was left in place for another 7 min before removal of the syringe. Animals were then euthanized and brains were immediately harvested and frozen for further tissue processing.

2.3. Brain processing and imaging

Frozen brains were cut in 50 μm slices using a Leica Cryostat CM1850 (Leica, Germany) and mounted on positively charged frosted microscope slides. Images of slices were taken with a Zeiss SteREO Lumar.V12 microscope (Zeiss, Germany) with a Zeiss NeoLumar S 1.5 x FWD 30 mm objective lens. Images were taken with an exposure time of 350 ms in a 8 bit grey format. Images were 1388 × 1040 pixels with a black value of 0, white value of 0.0625 and gamma value of 0.45. Finally the images were analyzed using programs written in MATLAB (MathWorks, Natick, MA, USA).

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