Published in final edited form as:

Methods Enzymol. 2012 ; 508: 109–130. doi:10.1016/B978-0-12-391860-4.00006-9.

Multistage Nanoparticles for Improved Delivery into Tumor Tissue

Triantafyllos Stylianopoulos^{*}, Cliff Wong[†], Mouni G. Bawendi[†], Rakesh K. Jain[‡], and Dai Fukumura[‡]

^{*}Department of Mechanical and Manufacturing Engineering, University of Cyprus, Nicosia, Cyprus

[†]Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts, USA

[‡]Edwin L. Steele Laboratory, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts, USA

Abstract

The enhanced permeability and retention (EPR) effect has been a key rationale for the development of nanoscale carriers to solid tumors. As a consequence of EPR, nanotherapeutics are expected to improve drug and detection probe delivery, have less adverse effects than conventional chemotherapy, and thus result in improved detection and treatment of tumors. Physiological barriers posed by the abnormal tumor microenvironment, however, can hinder the homogeneous delivery of nanomedicine in amounts sufficient to eradicate cancer. To effectively enhance the therapeutic outcome of cancer patients by nanotherapeutics, we have to find ways to overcome these barriers. One possibility is to exploit the abnormal tumor microenvironment for selective and improved delivery of therapeutic agents to tumors. Recently, we proposed a multistage nanoparticle delivery system as a potential means to enable uniform delivery throughout the tumor and improve the efficacy of anticancer therapy. Here, we describe the synthesis of a novel multistage nanoparticle formulation that shrinks in size once it enters the tumor interstitial space to optimize the delivery to tumors as well as within tumors. Finally, we provide detailed experimental methods for the characterization of such nanoparticles.

1. Introduction

Systemic administration of nanomedicines to solid tumors is a three-step process (Jain, 1987a,b, 2001; Jain and Baxter, 1988). Nanoparticles have to first travel through the blood vessels and reach the tumor tissue. Subsequently, the nanoparticles need to cross the tumor vessel walls and finally travel through the interstitial space to attack cancer cells. For optimal delivery and enhanced therapeutic efficacy, nanoscale drugs must have long circulation times in the bloodstream, and effective transport across the tumor vessel walls, as well as through the tumor interstitial space (Chauhan *et al.*, 2011; Jain and Stylianopoulos, 2010).

1.1. Considerations for vascular transport of nanoparticles

Provided the therapeutic agent is not toxic to normal organs, it is reasonable to prolong blood circulation time to allow longer interaction with the tumor tissue. Blood circulation time is determined by the clearance rate through renal excretion and interactions with the reticuloendothelial system in the liver and the spleen. Relatively, small nanoparticles with hydrodynamic diameter smaller than 6 nm have a blood half-life < 600 min and are rapidly cleared by the kidney, whereas the blood half-life of larger particles might exceed 1000 min (Choi *et al.*, 2010, 2007; Longmire *et al.*, 2008; Petros and DeSimone, 2010; Popovic *et al.*,

2010). Clearance from the reticuloendothelial system might vary significantly among the different types of nanoparticles and can be controlled by surface modifications (Franzen and Lommel, 2009; Longmire *et al.*, 2008). Today, coating the surface of a nanoparticle with a layer of polyethyleneglycol (PEG) is a common practice. Attachment of PEG to the surface sterically stabilizes nanoparticles and results in a near neutral surface charge. PEGylation protects nanoparticles from opsonization by serum proteins and phagocytosis by Kupffer cells and hepatocytes (Klibanov *et al.*, 1990; Peracchia *et al.*, 1999; Storm *et al.*, 1995).

1.2. Considerations for transvascular transport of nanoparticles

Transport across the tumor vessel walls and through the tumor interstitial space is determined by the properties of the nanoparticle (size, shape, and charge) and the tumor architecture (Baish *et al.*, 2011; Jain, 1987a; Popovic *et al.*, 2010; Stylianopoulos *et al.*, 2010a,b). Tumor vessels are in general tortuous and nonuniformly distributed, typically exhibiting high vascular density in the periphery and lower in the center of the tumor (Chauhan *et al.*, 2011; Jain and Baxter, 1988; Vakoc *et al.*, 2009). In addition, many tumor vessels have an abnormal endothelial cell lining, detached pericytes, and an abnormally thick or thin basement membrane (Fig. 6.1; Jain, 1987a). As a result, the size of the pores of the vessel walls is heterogeneous. We have shown previously that the pore size ranges from 10 to 2000 nm in various tumors growing in animal models (Hashizume *et al.*, 2000; Hobbs *et al.*, 1998). Collectively, tumor vessels are nonuniformly leaky and have relatively larger pore size, on average, than normal vessels. This is the actual basis of the EPR effect.

Many normal vessels have a pore size in the range of 10 nm, much larger than the size of a chemotherapeutic molecule (<1 nm), but smaller than the size of most nanoparticle formulations. As a result, systemically administered chemotherapeutics are delivered effectively not only to tumors but also to normal tissues. Since chemotherapeutics are toxic to both cancer and normal cells, lack of preferential delivery results in significant adverse effects in normal tissues. On the contrary, a nanoparticle with size larger than the pore size of normal vessels could preferentially pass through the pores of tumor vessels and diminish the adverse effects.

1.3. Considerations for interstitial transport of nanoparticles

The hyperpermeability of tumor vessels combined with the absence of functional lymphatic vessels at the center of the tumor leads to a uniformly elevated interstitial fluid pressure (IFP) (Boucher and Jain, 1992; Boucher *et al.*, 1990, 1991; Gutmann *et al.*, 1992; Jain and Baxter, 1988; Less *et al.*, 1992; Roh *et al.*, 1991). IFP in many tumors might be as high as microvascular pressure (MVP), whereas in many cases, it can locally exceed MVP. This effect might diminish pressure gradients across the vessel walls and in the tumor interstitial space (Baxter and Jain, 1989, 1990; Jain and Baxter, 1988; Jain *et al.*, 2007). In such a case, the main mechanism of drug transport is diffusion, which can be a very slow process, particularly for large nanoparticles (Chary and Jain, 1989; Nugent and Jain, 1984; Pluen *et al.*, 2001; Ramanujan *et al.*, 2002).

In addition, in many tumors there is an accumulation of fibrillar collagen types I and III in the interstitial space, presumably due to increased activity of factors, such as transforming growth factor- β (TGF- β), that might stiffen the extracellular matrix and induce fibrosis (Branton and Kopp, 1999; Brown *et al.*, 2003; Butcher *et al.*, 2009; Diop-Frimpong *et al.*, 2011; Egeblad *et al.*, 2010; Huijbers *et al.*, 2010; Kauppila *et al.*, 1998; McKee *et al.*, 2006; Netti *et al.*, 2000). Increased fibrillar collagen may hinder the diffusion of nanoparticles in tumors. In particular, it has been shown that, in collagen-rich tumors, nanoparticles with hydrodynamic diameter larger than 60 nm might not be able to effectively penetrate the collagen network that comprises the tumor interstitium (Brown *et al.*, 2003; Diop-Frimpong

et al., 2011; McKee *et al.*, 2006; Netti *et al.*, 2000; Pluen *et al.*, 2001). These particles will cross the leaky tumor vessels, but because they cannot diffuse effectively, they are likely to accumulate in perivascular regions and cause only local effects (Yuan *et al.*, 1994). One approach to improve the delivery is to modify the collagen matrix (Brown *et al.*, 2003; Diop-Frimpong *et al.*, 2011; McKee *et al.*, 2006; Netti *et al.*, 2000). In the next section, we discuss an alternative approach—a multistage delivery system to overcome this challenge.

1.4. Multistage nanoparticle systems to optimize drug delivery

Currently, FDA-approved anticancer nanoparticles include Doxil® (ovarian cancers, metastatic breast cancers, and HIV-related Kaposi's sarcomas) and Abraxane® (metastatic breast cancers). The size of these nanoparticles is ~100 nm in diameter. Both Doxil® and Abraxane® are associated with significant reduction in the adverse effects as compared to conventional chemotherapy. However, the survival benefit is relatively modest presumably due to inadequate delivery to tumors (Gordon *et al.*, 2001; Gradishar *et al.*, 2005; Jain and Stylianopoulos, 2010). For optimal efficacy, a therapeutic agent must reach tumors in amounts sufficient to destroy cancer cells, but at the same time should not cause significant adverse effects in normal tissues. In general, relatively small size nanoparticles have higher transvascular and interstitial transport (Popovic *et al.*, 2010). However, small particles might have a shorter blood half-life and be delivered to normal tissues, causing adverse effects. Therefore, the size of the particle needs initially to be relatively large to achieve high blood half-life and selective extravasation, but once it enters the tumor interstitium, it needs to have a small size for effective and uniform penetration. These contradicting/opposing demands for the size of nanomedicines have led to the development of multistage nanoparticles that are able to change their size in response to stimuli from the tumor microenvironment.

We describe the synthesis of a multistage nanoparticle delivery system which initially has a size of ~100 nm, but when it reaches the tumor, it shrinks its size to a ~10 nm particle that can more effectively diffuse throughout the tumor interstitial space (Fig. 6.2; Wong *et al.*, 2011). To achieve this size change in tumor tissues, the methodology makes use of matrix metalloproteinase (MMP) enzymes that are overexpressed in the microenvironment of many tumors to degrade the core of a ~100-nm gelatin nanoparticle so that smaller ~10 nm diagnostic or therapeutic agents can be released from its surface. Methods to assess the size change of the nanoparticle upon MMP activation, such as gel filtration chromatography (GFC), fluorescence correlation spectroscopy (FCS), as well as diffusion measurements in collagen gels, are described in detail. *In vivo* techniques based on intravital microscopy (IVM) that are used to measure the delivery of multistage nanoparticles in animal models are also discussed. These techniques include systemic and local administration of the multistage nanoparticles in human tumor xenografts to estimate blood half-life, systemic delivery, and interstitial transport.

2. Synthesis of Gelatin-Quantum Dot Nanoparticles (QDGeINP)

2.1. Synthesis of gelatin nanoparticles

Reagents used for the synthesis of the gelatin particles include:

1. Gelatin type A ~175 bloom from porcine skin (Sigma).
2. Acetone (EMD Chemicals).
3. Deionized water (Thermo Scientific® Barnstead Nanopure).
4. HCl solution, 1 N (Sigma).
5. Glutaraldehyde solution (Grade I, 50%) (Sigma).

6. Glycine solution, 1 M (Sigma).
7. 10 × PBS (EMD Chemicals).

To synthesize the gelatin nanoparticles, we perform the following steps (Wong *et al.*, 2011; Fig. 6.3): 0.625 g of gelatin is added to 12.5 ml of deionized water and heated to 40 °C in a 100-ml round-bottom flask until fully dissolved. The flask is then removed from heat and 12.5 ml of acetone are added to the solution at 6.0 ml/min using a PHD2000 syringe pump (Harvard Apparatus, Holliston, MA, USA) while stirring at 300 rpm. The high-molecular weight gelatin fraction will begin precipitating onto the bottom of the round-bottom flask. The stirring is turned off when the acetone addition is complete. Exactly 1 min after the addition of acetone, the supernatant containing the low-molecular weight gelatin fraction is discarded. Deionized water (12.5 ml) is added to the remaining precipitate and heated again to 40 °C, until complete dissolution. Half the solution is removed and the pH of this half is adjusted to 2.7 with a 1 N HCl solution while recording the exact volume needed. The pH is monitored using a Thermo Orion PerpHecT Sure-Flow pH electrode (Thermo Fisher Scientific, Asheville, NC, USA). This electrode minimizes clogging but causes contamination with the pH electrode solution. Therefore, this half of the gelatin solution is discarded after recording the amount of HCl needed to change the pH to 2.7. This exact volume of 1 N HCl solution is added to the remaining half of gelatin solution to adjust the pH to 2.7 and used for the next step. Under constant stirring at 600 rpm and 40 °C, 20.75 ml of acetone are added at 1 ml/min using the syringe pump. Following addition of ~17 ml of acetone, the solution appears cloudy white. After the acetone addition is complete, 30 µl of 50% glutaraldehyde solution (Grade I) diluted in 1 ml acetone is added to the gelatin solution at 0.05 ml/min to crosslink the particles. Afterward, the solution is kept at 40 °C and stirred at 600 rpm for 7.5 h. The acetone is then evaporated slowly at 25 °C to a final volume of 5–6 ml using a rotary evaporator. The remaining solution is first filtered through a 0.45-µm syringe filter to prevent clogging and then through a 0.2-µm syringe filter for finer size selection. A 1 M glycine solution (0.2 ml) is added, and the solution is stored overnight at 4 °C. The glycine should quench any remaining reactive glutaraldehyde groups. GFC is used subsequently to purify the nanoparticles. We have also tried centrifugation and centrifugal filters to purify the nanoparticles, but they both result in an increase in the size of the particles. A 1 ml solution of the gelatin particles is injected into a Superose™ 6 GL 10/300 column (GE Healthcare, Piscataway, NJ, USA) with 1 × PBS as the mobile phase. The peak eluting at the void volume is collected with 0.5 ml fractions. This procedure is repeated one more time, and the first concentrated fractions from both GFC runs are combined. This 1 ml solution is then taken to the next step for quantum dot (QD) conjugation and PEGylation.

2.2. Synthesis of gelatin-quantum dot nanoparticles

Reagents used for the synthesis of the QDGeNP include:

1. Gelatin nanoparticles (see Section 2.1).
2. Hydroxysulfosuccinimide sodium salt (sulfo-NHS) (Sigma).
3. *N*-(3-Dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC) (Sigma).
4. Methoxypolyethylene glycol amine 5000 (mPEG-amine 5 kDa) (Sigma).
5. HEPES buffer (EMD Chemicals).
6. Qdot® 565 ITK™ amino (PEG) quantum dot (Invitrogen).

The 1 ml gelatin nanoparticle (NP) solution is mixed with 20 µl of 8 µM Qdot® 565 ITK™ amino (PEG) QDs in a 7-ml glass vial. The solution is gently stirred at 130 rpm for 30 min. High stir speeds result in particle aggregation. Afterwards, the pH is changed to 5 using 1 N

HCl solution and then immediately adjusted to pH 6 using 1 N NaOH. The pH is checked by removing 1 μ l of the solution using a 10 μ l micropipette and applying it onto a pH strip. Stirring is continued for 30 min. EDC (0.4 mg, 2.1 μ mol) and sulfo-NHS (0.4 mg, 1.9 μ mol) are dissolved in 50 μ l of deionized water and then added to the gelatin NP/QD mixture. The reaction proceeds for 3 h. Subsequently, mPEG-amine 5 kDa (20 mg, $\sim$ 4 μ mol) dissolved in 50 μ l of deionized water is added to the gelatin NP/QD solution. Then, another solution of EDC (0.4 mg) and sulfo-NHS (0.4 mg) dissolved in 50 μ l of deionized water is added. After 2 h of reaction, the pH is adjusted to 8 using 1 N NaOH, and the stirring continues for 1 h. A 1 M glycine solution (50 μ l) is added to quench the reaction. After 30 min, the resulting solution is filtered through a 0.2 μ m syringe filter to remove any aggregates. This solution is then purified using GFC with the Superose™ 6 column. The mobile phase for the GFC is 50 mM HEPES buffer at pH 7.5 for subsequent *in vitro* characterization of the QDGelNPs or PBS at pH 7.4 for subsequent *in vivo* experiments. The product eluting at the void volume is collected in 0.5 ml fractions, and the first concentrated fraction is used for further experiments.

3. Kinetics of QDGelNP Degradation

To measure the kinetics of MMP-2-induced QD release, QDGelNP are incubated with MMP-2 for various time intervals. QDGelNP (0.1 mg) in 50 mM HEPES and 2 mM CaCl₂ are incubated with 230 ng of MMP-2 for a maximum of 12 h at 37 °C. After incubation, an EDTA solution (200 mM) is added to obtain a final concentration of 20 mM EDTA to inhibit further MMP-2 cleavage. The samples before and after incubation are analyzed by GFC, with a Superose™ 6 GL 10/300 column on an Agilent 1100 series HPLC with an in-line degasser, autosampler, diode array detector, and fluorescence detector (Roseville, CA, USA). The injection volume is 45 μ l and the mobile phase is 1 $\times$ PBS (pH 7.4) at a flow rate of 0.5 ml/min. The GFC chromatograms are detected by fluorescence, with 250 nm excitation wavelength and 565 nm emission wavelength, which permits the measurement of only the QDs' elution profile. To normalize for changes in the QD fluorescence intensity over time and scattering effects, the integrated intensities of the chromatograms from 13 to 38 min are set to unity. The percentage of QDs that are released over time is then calculated. The peak corresponding to the free QDs is integrated (from 18 to 38 min) and then corrected for peak tailing from the peak eluting at void volume. The correction for peak tailing is done by supposing that for a certain integrated area of the peak at void volume, a fixed percentage of that integrated area will be added to the peak for individual QDs. We obtain the fixed percentage of 26.5% using QDGelNP before cleaving, which should elute completely at the void volume if not for peak tailing. A typical kinetic curve of gelatin degradation and QD release is shown in Fig. 6.4.

4. Multiphoton Fluorescence Correlation Spectroscopy

FCS is an accurate method to measure the diffusion coefficient of nanoparticles in a small focal volume allowing for two-component or anomalous diffusion measurements. It can be used with either confocal or multiphoton laser scanning microscopy, but it is restricted to low-concentration samples (Alexandrakis *et al.*, 2004; Schwille *et al.*, 1999). For multiphoton laser scanning microscopy, the laser is usually set to 800–900 nm, and it is aligned through the Pockel's cell. Photomultiplier tubes (PMTs) are installed along with the appropriate band-pass filters to select the emission spectra of interest. One PMT is used for autocorrelation analysis, whereas two PMTs are required for cross-correlation. Fluctuations in fluorescence intensity are recorded as fluctuations in the photocurrent of the PMT. The PMTs are connected to a digital correlator card that performs the autocorrelation of the fluorescence intensity signal. The resulting autocorrelation curve is displayed on a computer that allows control of the data acquisition time and analysis of the autocorrelation curves.

Usually, a 40× or 60× water emission objective is used with the microscope, and the whole system is controlled by suitable commercial software.

For *in vitro* studies, the samples of interest can be prepared by sealing a silicone perfusion chamber over a 22 × 50-mm glass coverslip. It is recommended to treat the coverslip surface with a 1% (w/v) casein in PBS solution to prevent nonspecific binding of nanoparticles to the surface of the glass. For *in vivo* diffusion measurements, the particles can be injected directly to the tumor microenvironment as described in Section 7.1. The concentration of the nanoparticles, the excitation power, and the acquisition time should be adjusted properly so that the diffusion measurements are not influenced by these parameters. The FCS technique is based on the analysis of fluctuations of fluorophores in the focal volume. Lower concentration of nanoparticles in the focal volume increases the intensity fluctuations and results in improvement in the quality of the FCS data (unless the signal intensity from the lower concentration sample becomes too dim). High excitation power can cause photobleaching of the particles, which might become more significant for longer acquisition times. We recommend that nanoparticle solutions of varying concentrations are tested, and the excitation power and acquisition time are tuned to get optimal conditions. We also recommend that the number of nanoparticles in the focal volume be larger than 80 but should not exceed 1000. Usually the excitation power is less than 25 mW, depending on the optical properties of the nanoparticles, and the acquisition time is from 60 to 90 s (Alexandrakis *et al.*, 2004; Stylianopoulos *et al.*, 2010b). Because FCS measurements are sensitive to random noise caused by aggregates or other sources, we suggest that 5–10 correlation curves are obtained from the same spot in the sample and their values are averaged. It is also crucial to place a cover over the sample, stage, and objective during the measurements to minimize ambient light noise.

To extract the apparent diffusion coefficient, D , the correlation curves are best-fitted to diffusion equations. For isotropic single-component diffusion, the mean-square displacement, $\langle r^2 \rangle$, of the particles is proportional to time, t : $\langle r^2 \rangle = 6Dt$. The equation for the correlation function, G , is:

$$G(t) = \frac{1}{N} \left(1 + \frac{t}{\tau_d} \right)^{-1} \left(1 + \frac{t}{K^2 \tau_d} \right)^{-1/2} + G_{\text{inf}}, \quad (6.1)$$

where N is the total number of particles in the focal volume; $K = z_0/r_0$ with z_0 and r_0 the axial and radial dimension of the focal volume, respectively; the characteristic time $\tau_d = r_0^2/8D$; and G_{inf} is an offset. The experimental data of $G(t)$ versus t is then fit to the above equation to determine the values of N , D , and G_{inf} .

When diffusion measurements are performed *in vivo* or in tissue phantoms (e.g., collagen gels), nanoparticles might exhibit a two-component diffusion resulting from the heterogeneous structure of the tissue. The slow component corresponds to the viscous phase of the tissue, characterized by high collagen density. The fast component corresponds to the aqueous phase, which is usual in the distant regions from the collagen fibers (Alexandrakis *et al.*, 2004; Chauhan *et al.*, 2009). For two-component diffusion, the correlation function takes the form:

$$G(t) = \frac{1}{N} \phi_1 \left(1 + \frac{t}{\tau_1} \right)^{-1} \left(1 + \frac{t}{K^2 \tau_1} \right)^{-1/2} + \frac{1}{N} \phi_2 \left(1 + \frac{t}{\tau_2} \right)^{-1} \left(1 + \frac{t}{K^2 \tau_2} \right)^{-1/2}, \quad (6.2)$$

where ϕ_1 and ϕ_2 ($\phi_2 = 1 - \phi_1$) are the population fractions of each component of the diffusion, $\tau_1 = r_0^2/8D_1$ and $\tau_2 = r_0^2/8D_2$ are the two characteristic times, and D_1 and D_2 are the two components of the diffusion coefficient. Regression of Eq. (6.2) to the experimentally obtained correlation curves determines the values of ϕ_1 , D_1 , and D_2 .

For anomalous subdiffusion, the mean-square displacement of the particles is proportional to some power of time ($0 < \alpha < 1$), that is, $\langle r^2 \rangle = 6 \alpha t$. In this case, the apparent diffusion coefficient depends on time and is given by the quantity $D(t) = \langle r^2 \rangle / 6t = \alpha t^{-1}$. Anomalous diffusion is a generalization of normal diffusion (which occurs when $\alpha = 1$), and the autocorrelation functions are derived by replacing t_d in Eq. (6.1) with $(t_d)^\alpha$ to yield

$$G(t) = \frac{1}{N} \left(1 + \left(\frac{t}{\tau_d} \right)^\alpha \right)^{-1} \left(1 + \frac{1}{K^2} \left(\frac{t}{\tau_d} \right)^\alpha \right)^{-1/2} + G_{\text{inf}}. \quad (6.3)$$

Finally, the dimensions of the focal volume, r_0 and z_0 , can be calculated by obtaining correlation curves of particles of known diffusion coefficient and fitting Eq. (6.1) to them. Such a calibration can be performed by obtaining correlation curves of fluorescent latex spheres of known diameter in water whose diffusion coefficient is given by the Stokes–Einstein relationship. Correlation curves of such particles can be measured and then Eq. (6.1) can be used to fit for K and r_0 with known D . Subsequently, one can use these values of K and r_0 to perform diffusion measurements of nanoparticles of interest.

5. In Vitro Measurements of Interstitial Penetration

In vitro measurements of the interstitial penetration of nanoparticles can be performed with collagen gels that mimic the interstitial space of tumors. The gels are usually prepared with rat tail collagen I (BD Biosciences, San Jose, CA, USA) with the addition of sodium hydroxide to convert the pH to alkaline and induce collagen gelation (Chauhan *et al.*, 2009; Ramanujan *et al.*, 2002; Stylianopoulos *et al.*, 2010b). With the addition of sodium hydroxide to the collagen, the solution is vortexed immediately to allow uniform distribution of sodium hydroxide, and it is placed into a microslide capillary tube (Vitrocom no. 2540, Mountain Lakes, NJ, USA). The capillary tube is filled partially, and the collagen is incubated overnight at 37 °C. A 20 μ l mixture of QDGelNPs, either before or after incubation with MMP-2, is added to the capillary tube in contact with the free surface of the collagen gel. The particles penetrate the collagen structure, and depending on their size, they travel a different distance in the gel. The penetration distance can be measured with multiphoton laser scanning microscopy, and processing of the images with ImageJ or other image processing software. The diffusion of the particles in the tube can be considered one-dimensional and is governed by the following one-dimensional model:

$$C(x, t) \propto \text{erfc} \left(\frac{x}{2 \sqrt{D_{\text{eff}} t}} \right), \quad (6.4)$$

where erfc is the complementary error function. Fitting this equation to the concentration/intensity profile of the nanoparticles in the capillary tube for a given diffusion time, t , one can get the value of the effective diffusion coefficient D_{eff} . The time, t , in which particles are allowed to diffuse in the gel depends on how fast they move, and it usually ranges from 6 to 12 h.

6. InSitu Zymography

Before performing any *in vivo* studies, it is crucial to identify a tumor model that exhibits high MMP-2 activity, and thus, it can more easily cause degradation of the QDGeINPs. MMP-2 activity between different tumor models can be compared with *in situ* zymography. According to this technique (Mook *et al.*, 2003), a 1% (w/v) low gelatin temperature agarose in PBS is prepared and 4,6-diamidino-2-phenylindole (DAPI) is added to reach a final concentration of 1.0 µg/ml. The agarose solution is then mixed with a 1.0 mg/ml solution of DQ gelatin at a 10:1 ratio. DQ gelatin contains quenched FITC, which becomes unquenched under MMP degradation of the gelatin. The amount of degradation is related to the FITC intensity when imaged on a confocal microscope. Forty microliters of the agarose solution that contains DQ gelatin and DAPI are placed on top of cryostat sections (10 µm thick) of the tumors of interest and enclosed with a coverslip. Following solidification of the agarose at 4 °C, the gel and tumor section are incubated for 2 h at room temperature. Subsequently, confocal imaging is performed for DAPI and FITC. DAPI stains the cell nuclei, and since MMP-2 is secreted by cells, regions with high cellular density are expected to have high MMP activity, which is shown as increased FITC intensity. Fluorescence of FITC is detected by excitation at 460–500 nm and emission at 512–542 nm, and DAPI is detected by excitation at 340–380 nm and emission at 425 nm. Comparison of the FITC intensity for various tumor types can reveal the tumors that exhibit the highest MMP-2 activity.

7. Intravital Microscopy—*In vivo* Measurements

In vivo validation of the multistage nanoparticle system is essential to prove its capability for long circulation times and deep penetration into tumor tissue. Experimental protocols involve the use of IVM to measure (i) diffusion of the particles in the interstitial space of tumors, (ii) intratumoral delivery following systemic administration, and (iii) blood half-life (Fukumura *et al.*, 2010; Jain *et al.*, 2002). IVM is a noninvasive technique that allows imaging at multiple time intervals and has increased imaging depth of up to 400 µm with minimal photodamage. Confocal laser scanning microscopy and multiphoton laser scanning microscopy are the two most common techniques of IVM. The latter offers significant advancements, such as improved signal-to-noise ratio and improved imaging depth (Brown *et al.*, 2001; Padera *et al.*, 2002). IVM requires four components: (i) a transparent window placed in the animal model to allow visualization, (ii) the optically active nanoparticles that serve as contrast agents and can be detected by the microscope, (iii) a laser microscope accompanied by a detection system, and (iii) mathematical models and computer programs that can be used to process the images and measure parameters of interest.

Animal models that are most often used in cancer studies are mice with compromised immune systems (e.g., nude or severe combined immunodeficient (SCID) mice). Transparent windows might be placed with a surgical operation on the dorsal skin, the mammary fat pad, or the brain of the mouse depending on the origin of the tumor of interest (Jain *et al.*, 2010). Placement of a window in other organs such as liver or pancreas is also feasible (Jain *et al.*, 2002). The tumor source can be a suspension of cancer cells or a piece of tumor tissue that are implanted in the region where the tumor is expected to grow. Usually, the transparent window is placed first and then the cancer cells or the tumor fragment is implanted. However, if the tumor has a low chance of growing, it is common to reverse the order so that windows will be placed only to the mice whose tumor has grown. In principle, it is also possible to image spontaneously occurring tumors (Hagendoorn *et al.*, 2006; Kim *et al.*, 2010). Finally, to perform experiments, the tumor has to be vascularized, and thus, it is left to grow to a size of ~5 mm. The time that takes the tumor to grow to that size might vary from a few days to weeks, depending on the tumor growth rate and the implantation procedure (e.g., number of cancer cells implanted).

7.1. Interstitial transport measurements of multistage nanoparticles

To investigate if the MMP-2 activity of a tumor tissue is sufficient to cause degradation of the gelatin nanoparticles, direct injection of the particles into the tumor and subsequent imaging of their distribution is performed. The 100 nm gelatin particles have to be equipped with a smaller (10 nm) optically active agent to be detectable by the microscope. QDs can be used as a model system because they have high resistance to photo and chemical degradation, narrow photoluminescence spectra, broad excitation spectral windows, and large two-photon absorption cross sections (Allen *et al.*, 2010; Popovic *et al.*, 2010; Stroh *et al.*, 2005). In addition, a control group of nondegradable QDs with the same size as the gelatin particles (100 nm) but with a different emission wavelength from the 10 nm QDs is required. A solution of both types of nanoparticles is made and a very small volume of it ($\sim 1 \mu\text{l}$) is injected directly into the tumor of an anesthetized mouse, a technique known as intratumoral microinjection.

The injection has to be done very slowly to eliminate pressure gradients so that all injected particles stay at the injection site and do not move to the surrounding tumor tissue by convection. The injection rate is on the order of $0.05 \mu\text{l}/\text{min}$. With a commercial needle, it is not feasible to inject such a low volume of nanoparticles at these slow rates. Instead, a needle made by a glass tube of $200 \mu\text{m}$ in diameter is used. The tube is initially heated in the middle and is pulled apart from its edges. Once the heated part melts, the tube breaks in two pieces and a “needle” with diameter on the order of a few micrometers is formed at the heated side of each piece. The tip of the needle is then carefully broken under a light microscope so that the lumen increases to $\sim 40 \mu\text{m}$ in diameter. The tube is then filled with the solution of the nanoparticles and connected with a plastic tube to a syringe filled with silicon oil. Silicon oil has a high viscosity, which makes the motion of the piston of the syringe more easily controllable. The glass tube penetrates the surface of the tumor and the particles are released at a depth of $300\text{--}500 \mu\text{m}$ from the surface. A light microscope to better control the injection point and a system to control the penetration depth of the glass tube are required for improved accuracy. With the completion of the injection, the mouse is ready for intravital imaging.

Imaging of the nanoparticles is performed using a multiphoton laser scanning microscope (Fukumura *et al.*, 2010). The microscope is equipped with two PMTs carrying different emission filters so that both particle types (QDGelNPs and control) can be simultaneously detected. Comparison of images taken at different time intervals can reveal how fast the particles diffuse away from the injection point, which is directly related to their size, as smaller particles diffuse faster than larger one (Fig. 6.5). FCS is also a method that can be used with multiphoton microscopy to measure the diffusion coefficient of QD nanoparticles *in vivo* (Alexandrakis *et al.*, 2004).

The emission intensity of different types of nanoparticles might be quite different, and thus, simultaneous imaging requires suitable calibration of the multiphoton system. Before performing *in vivo* imaging, a solution containing both types of particles is placed in a capillary tube and imaged to determine the right emission filters to be used and the PMT sensitivity. The bandwidth of each emission filter should not allow the signal from the other particles to enter the PMT. PMT sensitivity should be calibrated so that the intensity of both types of nanoparticles in the solution is the same. The photoluminescence intensity also depends on the power of the laser. For animal studies, the laser power usually does not exceed $500\text{--}600 \text{ mW}$. Higher power of laser might damage the tissue and distort the quality of the images. The images are usually taken as multiple three-dimensional stacks. The average or maximum intensity z -projections of the stacks are extracted using an image

processing software (e.g., ImageJ). Images of consecutive adjacent regions in the x and y directions are combined to form a mosaic of the entire injection site.

7.2. Delivery through the systemic circulation

Systemic administration of nanoparticles is most often performed with retro-orbital or tail-vein injection (Steel *et al.*, 2008). According to the retro-orbital injection, the mouse is anesthetized and 200 μ l of the nanoparticle solution are injected into the retrobulbar sinus. The needle, whose length should not exceed 0.5 in., is inserted at a 45° angle to the eye, lateral to the medial canthus, and through the conjunctival membrane. It passes behind the globe of the eye to enter the retrobulbar sinus. This is a technique that requires proper training, because it can severely injured the animal, but it requires less than 5 min if it is performed proficiently. A mouse should, however, receive no more than one injection per day for each eye, and there should be 1–2 days between repeated injections. The tail vein is an alternative to the retro-orbital injection route. It does not require anesthesia, is safer, and allows administration of larger amounts of nanoparticles, but it is more time-consuming. The animal is first placed on a heat pad to increase blood flow to the tail vein. It is then transferred to a holding device, which restrains the motion of the mouse, while allowing access to the tail vein. Starting from the tip of the tail and moving toward the body, the needle is inserted to the tail vein as parallel to the tail as possible. One should be able to see the needle entering the vein, or the solution going into the vein.

Following systemic administration, multiphoton imaging is carried out to form a mosaic of the entire tumor, and the intensity of the nanoparticles is measured. The normalized transvascular flux is calculated from the intensity of the particles using the equation (Brown *et al.*, 2001):

$$P_{\text{eff}} = \frac{J_t}{S_v(C_v - C)} = \lim_{t \rightarrow 0} \frac{\partial \int_{r=R}^{\infty} C(r) r dr}{\partial t (C_v - C) R}, \quad (6.5)$$

where J_t is the transvascular flux, S_v is the vessel surface area, C_v is the concentration of the probe in the vessel, C is the concentration of the probe immediately extravascular and is calculated based on the fluorescence intensity, P_{eff} is the effective permeability, t is the time after the initial image, r is the distance from the vessel central axis, and R is the vessel radius at that point along the vessel. The calculation is made as an average over the entire imaged volume for each tumor. Apart from transvascular flux, these images can show the interstitial penetration of the nanoparticles from the blood vessels and their distribution in the tumor interstitial space.

7.3. Blood circulation time

To measure the circulation time of nanoparticles, mice not bearing tumors may be used. Intravenous infusion of the particles is performed by retro-orbital or tail-vein injection. At different time intervals, starting 1 min before the injection of particles, a small quantity of blood (~13 μ l) is collected by means of a tail-vein nick and mixed with 3 μ l 50 mM EDTA. The samples are then imaged using multiphoton microscopy, and values of average intensities are extracted. Again, it is assumed that fluorescence intensity is proportional to the particle concentration. The clearance half-life is calculated by fitting a biexponential curve to the intensity data (Popovic *et al.*, 2010). The intensity data are characterized by a quick drop of the intensity at short times and a slow decrease at long times. The short time response is attributed to particle absorption by tissues, whereas the long time behavior is due to the clearance of the particles. Therefore, the clearance half-life corresponds to the long time exponent.

8. Conclusion and Perspective

In this chapter, we provide a detailed description of the methods for the formulation and characterization of a multistage nanoparticle delivery system that enables enhanced distribution to tumors. This multistage system is based on the enzymatic degradation of a ~ 100-nm gelatin carrier and the release from its surface of smaller diagnostic or therapeutic agents. We have shown the proof of principle of this strategy by administering the multistage nanoparticles directly into the tumor. The gelatinase enzymatic activity in HT1080 soft tissue sarcomas was sufficient to degrade the gelatin and release 10 nm QDs that were conjugated to the nanoparticle (Wong *et al.*, 2011). The next steps of the development of this multistage nanoparticle delivery system would be the optimization of the system via systemic administration and, ultimately, the creation of drug-loading multistage nanoparticles as well as the evaluation of their *in vivo* efficacy. These are necessary steps for the true evaluation of the hypothesis and for potentially moving toward clinical application.

It should be noted that there are other types of multistage nanoparticle delivery systems under development (Ferrari, 2005). For example, mesoporous silica particles function as carriers for the controlled release of therapeutic agents (Lu *et al.*, 2007; Tanaka *et al.*, 2010; Tasciotti *et al.*, 2008). These particles release their therapeutic load progressively while circulating in the bloodstream. Effort is also made for the optimal design of these particles to improve their margination and interaction with the abnormal tumor vasculature (Serda *et al.*, 2011). In addition, there are other promising strategies for cancer treatment, such as targeted nanoparticle delivery systems. These nanoparticles are equipped with peptides or targeting moieties that can selectively bind tumor endothelial cells or cancer cells (Davis *et al.*, 2010; Ruoslahti *et al.*, 2010), or enhance permeation to tumor tissue (Karmali *et al.*, 2009; Sugahara *et al.*, 2009). Ultimately, successful multistage nanoparticle systems are likely to combine different approaches and may even be customized for individual tumors and be adjustable during the course of treatment.

Acknowledgments

This chapter is based on the paper by Wong *et al.* (2011). This research was supported by US National Cancer Institute Grants R01-CA126642 (to R. K. J. and M. G. B.), P01-CA080124 (to R. K. J. and D. F.), R01-CA096915 (to D. F.); Federal Shared Proton Beam Program Income (to R. K. J.); National Center for Research Resources S10-RR027070 (to D. F.); Department of Defense Breast Cancer Research Program BC095991 (R. K. J.); and a FP7 Marie-Curie International Reintegration Grant PIRG08-GA-2010-276894 (to T. S.).

References

- Alexandrakis G, Brown EB, Tong RT, McKee TD, Campbell RB, Boucher Y, Jain RK. Two-photon fluorescence correlation microscopy reveals the two-phase nature of transport in tumors. *Nat Med.* 2004; 10:203–207. [PubMed: 14716306]
- Allen PM, Liu W, Chauhan VP, Lee J, Ting AY, Fukumura D, Jain RK, Bawendi MG. InAs(ZnCdS) quantum dots optimized for biological imaging in the near-infrared. *J Am Chem Soc.* 2010; 132:470–471. [PubMed: 20025222]
- Baish JW, Stylianopoulos T, Lanning RM, Kamoun WS, Fukumura D, Munn LL, Jain RK. Scaling rules for diffusive drug delivery in tumor and normal tissues. *Proc Natl Acad Sci USA.* 2011; 108:1799–1803. [PubMed: 21224417]
- Baxter LT, Jain RK. Transport of fluid and macromolecules in tumors. I. Role of interstitial pressure and convection. *Microvasc Res.* 1989; 37:77–104. [PubMed: 2646512]
- Baxter LT, Jain RK. Transport of fluid and macromolecules in tumors. II. Role of heterogeneous perfusion and lymphatics. *Microvasc Res.* 1990; 40:246–263. [PubMed: 2250603]

- Boucher Y, Jain RK. Microvascular pressure is the principal driving force for interstitial hypertension in solid tumors: Implications for vascular collapse. *Cancer Res.* 1992; 52:5110–5114. [PubMed: 1516068]
- Boucher Y, Baxter LT, Jain RK. Interstitial pressure gradients in tissue-isolated and subcutaneous tumors: Implications for therapy. *Cancer Res.* 1990; 50:4478–4484. [PubMed: 2369726]
- Boucher Y, Kirkwood JM, Opacic D, Desantis M, Jain RK. Interstitial hypertension in superficial metastatic melanomas in humans. *Cancer Res.* 1991; 51:6691–6694. [PubMed: 1742743]
- Branton MH, Kopp JB. TGF-beta and fibrosis. *Microbes Infect.* 1999; 1:1349–1365. [PubMed: 10611762]
- Brown EB, Campbell RB, Tsuzuki Y, Xu L, Carmeliet P, Fukumura D, Jain RK. In vivo measurement of gene expression, angiogenesis and physiological function in tumors using multiphoton laser scanning microscopy. *Nat Med.* 2001; 7:864–868. [PubMed: 11433354]
- Brown E, McKee T, diTomaso E, Pluen A, Seed B, Boucher Y, Jain RK. Dynamic imaging of collagen and its modulation in tumors in vivo using second-harmonic generation. *Nat Med.* 2003; 9:796–800. [PubMed: 12754503]
- Butcher DT, Alliston T, Weaver VM. A tense situation: Forcing tumour progression. *Nat Rev Cancer.* 2009; 9:108–122. [PubMed: 19165226]
- Chary SR, Jain RK. Direct measurement of interstitial convection and diffusion of albumin in normal and neoplastic tissues by fluorescence photobleaching. *Proc Natl Acad Sci USA.* 1989; 86:5385–5389. [PubMed: 2748592]
- Chauhan VP, Lanning RM, Diop-Frimpong B, Mok W, Brown EB, Padera TP, Boucher Y, Jain RK. Multiscale measurements distinguish cellular and interstitial hindrances to diffusion in vivo. *Biophys J.* 2009; 97:330–336. [PubMed: 19580771]
- Chauhan VP, Stylianopoulos T, Boucher Y, Jain RK. Delivery of molecular and nanomedicine to tumors: Transport barriers and strategies. *Annu Rev Chem Biomol Eng.* 2011; 2:281–298. [PubMed: 22432620]
- Choi HS, Liu W, Misra P, Tanaka E, Zimmer JP, Itty Ipe B, Bawendi MG, Frangioni JV. Renal clearance of quantum dots. *Nat Biotechnol.* 2007; 25:1165–1170. [PubMed: 17891134]
- Choi HS, Liu W, Liu F, Nasr K, Misra P, Bawendi MG, Frangioni JV. Design considerations for tumour-targeted nanoparticles. *Nat Nanotechnol.* 2010; 5:42–47. [PubMed: 19893516]
- Davis ME, Zuckerman JE, Choi CH, Seligson D, Tolcher A, Alabi CA, Yen Y, Heidel JD, Ribas A. Evidence of RNAi in humans from systemically administered siRNA via targeted nanoparticles. *Nature.* 2010; 464:1067–1070. [PubMed: 20305636]
- Diop-Frimpong B, Chauhan VP, Krane S, Boucher Y, Jain RK. Losartan inhibits collagen I synthesis and improves the distribution and efficacy of nanotherapeutics in tumors. *Proc Natl Acad Sci USA.* 2011; 108:2909–2914. [PubMed: 21282607]
- Egeblad M, Rasch MG, Weaver VM. Dynamic interplay between the collagen scaffold and tumor evolution. *Curr Opin Cell Biol.* 2010; 22:697–706. [PubMed: 20822891]
- Ferrari M. Cancer nanotechnology: Opportunities and challenges. *Nat Rev Cancer.* 2005; 5:161–171. [PubMed: 15738981]
- Franzen S, Lommel SA. Targeting cancer with ‘smart bombs’: Equipping plant virus nanoparticles for a ‘seek and destroy’ mission. *Nanomedicine (Lond).* 2009; 4:575–588. [PubMed: 19572822]
- Fukumura D, Duda DG, Munn LL, Jain RK. Tumor microvasculature and microenvironment: Novel insights through intravital imaging in pre-clinical models. *Microcirculation.* 2010; 17:206–225. [PubMed: 20374484]
- Gordon AN, Fleagle JT, Guthrie D, Parkin DE, Gore ME, Lacave AJ. Recurrent epithelial ovarian carcinoma: A randomized phase III study of pegylated liposomal doxorubicin versus topotecan. *J Clin Oncol.* 2001; 19:3312–3322. [PubMed: 11454878]
- Gradishar WJ, Tjulandin S, Davidson N, Shaw H, Desai N, Bhar P, Hawkins M, O'Shaughnessy J. Phase III trial of nanoparticle albumin-bound paclitaxel compared with polyethylated castor oil-based paclitaxel in women with breast cancer. *J Clin Oncol.* 2005; 23:7794–7803. [PubMed: 16172456]

- Gutmann R, Leunig M, Feyh J, Goetz AE, Messmer K, Kastenbauer E, Jain RK. Interstitial hypertension in head and neck tumors in patients: Correlation with tumor size. *Cancer Res.* 1992; 52:1993–1995. [PubMed: 1551128]
- Hagendoorn J, Tong R, Fukumura D, Lin Q, Lobo J, Padera TP, Xu L, Kuchelapati R, Jain RK. Onset of abnormal blood and lymphatic vessel function and interstitial hypertension in early stages of carcinogenesis. *Cancer Res.* 2006; 66:3360–3364. [PubMed: 16585153]
- Hashizume H, Baluk P, Morikawa S, McLean JW, Thurston G, Roberge S, Jain RK, McDonald DM. Openings between defective endothelial cells explain tumor vessel leakiness. *Am J Pathol.* 2000; 156:1363–1380. [PubMed: 10751361]
- Hobbs SK, Monsky WL, Yuan F, Roberts WG, Griffith L, Torchilin VP, Jain RK. Regulation of transport pathways in tumor vessels: Role of tumor type and microenvironment. *Proc Natl Acad Sci USA.* 1998; 95:4607–4612. [PubMed: 9539785]
- Huijbers IJ, Irvani M, Popov S, Robertson D, Al-Sarraj S, Jones C, Isacke CM. A role for fibrillar collagen deposition and the collagen internalization receptor endo180 in glioma invasion. *PLoS One.* 2010; 5:e9808. [PubMed: 20339555]
- Jain RK. Transport of molecules across tumor vasculature. *Cancer Metastasis Rev.* 1987a; 6:559–593. [PubMed: 3327633]
- Jain RK. Transport of molecules in the tumor interstitium: A review. *Cancer Res.* 1987b; 47:3039–3051. [PubMed: 3555767]
- Jain RK. Normalizing tumor vasculature with anti-angiogenic therapy: A new paradigm for combination therapy. *Nat Med.* 2001; 7:987–989. [PubMed: 11533692]
- Jain RK, Baxter LT. Mechanisms of heterogeneous distribution of monoclonal antibodies and other macromolecules in tumors: Significance of elevated interstitial pressure. *Cancer Res.* 1988; 48:7022–7032. [PubMed: 3191477]
- Jain RK, Stylianopoulos T. Delivering nanomedicine to solid tumors. *Nat Rev Clin Oncol.* 2010; 7:653–664. [PubMed: 20838415]
- Jain RK, Munn LL, Fukumura D. Dissecting tumour pathophysiology using intravital microscopy. *Nat Rev Cancer.* 2002; 2:266–276. [PubMed: 12001988]
- Jain, RK.; Munn, LL.; Fukumura, D. Intravital microscopy of normal and diseased tissues in mice. In: Goldman, RD.; Spector, DL., editors. *Live Cell Imaging: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 2010. p. 475–522.
- Jain RK, Tong RT, Munn LL. Effect of vascular normalization by antiangiogenic therapy on interstitial hypertension, peritumor edema, and lymphatic metastasis: Insights from a mathematical model. *Cancer Res.* 2007; 67:2729–2735. [PubMed: 17363594]
- Karmali PP, Kotamraju VR, Kastantin M, Black M, Missirlis D, Tirrell M, Ruoslahti E. Targeting of albumin-embedded paclitaxel nanoparticles to tumors. *Nanomedicine.* 2009; 5:73–82. [PubMed: 18829396]
- Kaupila S, Stenback F, Risteli J, Jukkola A, Risteli L. Aberrant type I and type III collagen gene expression in human breast cancer in vivo. *J Pathol.* 1998; 186:262–268. [PubMed: 10211114]
- Kim P, Chung E, Yamashita H, Hung KE, Mizoguchi A, Kuchelapati R, Fukumura D, Jain RK, Yun SH. In vivo wide-area cellular imaging by side-view endomicroscopy. *Nat Methods.* 2010; 7:303–305. [PubMed: 20228814]
- Klibanov AL, Maruyama K, Torchilin VP, Huang L. Amphipathic polyethyleneglycols effectively prolong the circulation time of liposomes. *FEBS Lett.* 1990; 268:235–237. [PubMed: 2384160]
- Less JR, Posner MC, Boucher Y, Borochovit D, Wolmark N, Jain RK. Interstitial hypertension in human breast and colorectal tumors. *Cancer Res.* 1992; 52:6371–6374. [PubMed: 1423283]
- Longmire M, Choyke PL, Kobayashi H. Clearance properties of nano-sized particles and molecules as imaging agents: Considerations and caveats. *Nanomedicine (Lond).* 2008; 3:703–717. [PubMed: 18817471]
- Lu J, Liong M, Sherman S, Xia T, Kovochich M, Nel AE, Zink JJ, Tamanoi F. Mesoporous silica nanoparticles for cancer therapy: Energy-dependent cellular uptake and delivery of paclitaxel to cancer cells. *Nanobiotechnology.* 2007; 3:89–95. [PubMed: 19936038]
- McKee TD, Grandi P, Mok W, Alexandrakis G, Insin N, Zimmer JP, Bawendi MG, Boucher Y, Breakefield XO, Jain RK. Degradation of fibrillar collagen in a human melanoma xenograft

- improves the efficacy of an oncolytic herpes simplex virus vector. *Cancer Res.* 2006; 66:2509–2513. [PubMed: 16510565]
- Mook OR, Van Overbeek C, Ackema EG, Van Maldegem F, Frederiks WM. In situ localization of gelatinolytic activity in the extracellular matrix of metastases of colon cancer in rat liver using quenched fluorogenic DQ-gelatin. *J Histochem Cytochem.* 2003; 51:821–829. [PubMed: 12754293]
- Netti PA, Berk DA, Swartz MA, Grodzinsky AJ, Jain RK. Role of extracellular matrix assembly in interstitial transport in solid tumors. *Cancer Res.* 2000; 60:2497–2503. [PubMed: 10811131]
- Nugent LJ, Jain RK. Extravascular diffusion in normal and neoplastic tissues. *Cancer Res.* 1984; 44:238–244. [PubMed: 6197161]
- Padera TP, Stoll BR, So PT, Jain RK. Conventional and high-speed intravital multiphoton laser scanning microscopy of microvasculature, lymphatics, and leukocyte-endothelial interactions. *Mol Imaging.* 2002; 1:9–15. [PubMed: 12920856]
- Peracchia MT, Fattal E, Desmaele D, Besnard M, Noel JP, Gomis JM, Appel M, d'Angelo J, Couvreur P. Stealth PEGylated polycyanoacrylate nanoparticles for intravenous administration and splenic targeting. *J Control Release.* 1999; 60:121–128. [PubMed: 10370176]
- Petros RA, DeSimone JM. Strategies in the design of nanoparticles for therapeutic applications. *Nat Rev Drug Discov.* 2010; 9:615–627. [PubMed: 20616808]
- Pluen A, Boucher Y, Ramanujan S, McKee TD, Gohongi T, di Tomaso E, Brown EB, Izumi Y, Campbell RB, Berk DA, Jain RK. Role of tumor-host interactions in interstitial diffusion of macromolecules: Cranial vs. subcutaneous tumors. *Proc Natl Acad Sci USA.* 2001; 98:4628–4633. [PubMed: 11274375]
- Popovic Z, Liu W, Chauhan VP, Lee J, Wong C, Greytak AB, Insin N, Nocera DG, Fukumura D, Jain RK, Bawendi MG. A nano-particle size series for in vivo fluorescence imaging. *Angew Chem Int Ed Engl.* 2010; 49:8649–8652. [PubMed: 20886481]
- Ramanujan S, Pluen A, McKee TD, Brown EB, Boucher Y, Jain RK. Diffusion and convection in collagen gels: Implications for transport in the tumor interstitium. *Biophys J.* 2002; 83:1650–1660. [PubMed: 12202388]
- Roh HD, Boucher Y, Kalnicki S, Buchsbaum R, Bloomer WD, Jain RK. Interstitial hypertension in carcinoma of uterine cervix in patients: Possible correlation with tumor oxygenation and radiation response. *Cancer Res.* 1991; 51:6695–6698. [PubMed: 1742744]
- Ruoslahti E, Bhatia SN, Sailor MJ. Targeting of drugs and nanoparticles to tumors. *J Cell Biol.* 2010; 188:759–768. [PubMed: 20231381]
- Schwille P, Haupts U, Maiti S, Webb WW. Molecular dynamics in living cells observed by fluorescence correlation spectroscopy with one- and two-photon excitation. *Biophys J.* 1999; 77:2251–2265. [PubMed: 10512844]
- Serda RE, Godin B, Blanco E, Chiappini C, Ferrari M. Multi-stage delivery nano-particle systems for therapeutic applications. *Biochim Biophys Acta.* 2011; 1810:317–329. [PubMed: 20493927]
- Steel CD, Stephens AL, Hahto SM, Singletary SJ, Ciavarrá RP. Comparison of the lateral tail vein and the retro-orbital venous sinus as routes of intravenous drug delivery in a transgenic mouse model. *Lab Anim (NY).* 2008; 37:26–32. [PubMed: 18094699]
- Storm G, Belliot SO, Daemen T, Lasic DD. Surface modification of nanoparticles to oppose uptake by the mononuclear phagocyte system. *Adv Drug Deliv Rev.* 1995; 17:31–48.
- Stroh M, Zimmer JP, Duda DG, Levchenko TS, Cohen KS, Brown EB, Scadden DT, Torchilin VP, Bawendi MG, Fukumura D, Jain RK. Quantum dots spectrally distinguish multiple species within the tumor milieu in vivo. *Nat Med.* 2005; 11:678–682. [PubMed: 15880117]
- Stylianopoulos T, Diop-Frimpong B, Munn LL, Jain RK. Diffusion anisotropy in collagen gels and tumors: The effect of fiber network orientation. *Biophys J.* 2010a; 99:3119–3128. [PubMed: 21081058]
- Stylianopoulos T, Poh MZ, Insin N, Bawendi MG, Fukumura D, Munn LL, Jain RK. Diffusion of particles in the extracellular matrix: The effect of repulsive electrostatic interactions. *Biophys J.* 2010b; 99:1342–1349. [PubMed: 20816045]

- Sugahara KN, Teesalu T, Karmali PP, Kotamraju VR, Agemy L, Girard OM, Hanahan D, Mattrey RF, Ruoslahti E. Tissue-penetrating delivery of compounds and nanoparticles into tumors. *Cancer Cell*. 2009; 16:510–520. [PubMed: 19962669]
- Tanaka T, Mangala LS, Vivas-Mejia PE, Nieves-Alicea R, Mann AP, Mora E, Han HD, Shahzad MM, Liu X, Bhavane R, Gu J, Fakhoury JR, et al. Sustained small interfering RNA delivery by mesoporous silicon particles. *Cancer Res*. 2010; 70:3687–3696. [PubMed: 20430760]
- Tasciotti E, Liu X, Bhavane R, Plant K, Leonard AD, Price BK, Cheng MM, Decuzzi P, Tour JM, Robertson F, Ferrari M. Mesoporous silicon particles as a multistage delivery system for imaging and therapeutic applications. *Nat Nanotechnol*. 2008; 3:151–157. [PubMed: 18654487]
- Vakoc BJ, Lanning RM, Tyrrell JA, Padera TP, Bartlett LA, Stylianopoulos T, Munn LL, Tearney GJ, Fukumura D, Jain RK, Bouma BE. Three-dimensional microscopy of the tumor microenvironment in vivo using optical frequency domain imaging. *Nat Med*. 2009; 15:1219–1223. [PubMed: 19749772]
- Wong C, Stylianopoulos T, Cui J, Martin J, Chauhan VP, Jiang W, Popovic Z, Jain RK, Bawendi MG, Fukumura D. Multistage nanoparticle delivery system for deep penetration into tumor tissue. *Proc Natl Acad Sci USA*. 2011; 108:2426–2431. [PubMed: 21245339]
- Yuan F, Leunig M, Huang SK, Berk DA, Papahadjopoulos D, Jain RK. Microvascular permeability and interstitial penetration of sterically stabilized (stealth) liposomes in a human tumor xenograft. *Cancer Res*. 1994; 54:3352–3356. [PubMed: 8012948]

Figure 6.1.

Contrary to normal vessels (right), tumor vessels (left) might have an abnormal endothelial cell (EC) lining, an abnormally thick or thin basement membrane (BM), and detached or absent pericytes (PC). As a result, nanoparticles can more easily pass through the tumor vessel wall.

Figure 6.2.

Schematic representation of the multistage nanoparticle delivery system. The initial 100-nm gelatin particle accumulates preferentially around leaky vessels in tumor tissue. Because of its large size, the 100-nm particle cannot penetrate the dense collagen matrix of the interstitial space. MMP-2 activity can degrade the gelatin core of the 100-nm particle and release smaller 10-nm particles from its surface. These particles can penetrate deep into the tumor due to their small size.

Figure 6.3.
Stages of QDGeINP synthesis.

Figure 6.4.
Kinetics of gelatin degradation and release of quantum dots (Wong *et al.*, 2011).

Figure 6.5.

(A) Images of QDGeINPs and silica quantum dots (SilicaQDs) at 1, 3, and 6 h after intratumoral coinjection into HT1080 tumor xenograft. (B) Relative fluorescence intensity of the corresponding images from panel (A). QDGeINPs and SilicaQDs have the same initial size of 100 nm. Degradation of gelatin by MMP-2 results in 10 nm QD particles that travel faster in the tumor interstitial space. Scale bar: 100 μm (Wong *et al.*, 2011).