Journal of Controlled Release 260 (2017) 124–133

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Photoactivatable fluorescent probes reveal heterogeneous nanoparticle permeation through biological gels at multiple scales

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ARTICLE INFO

Keywords:
Photoactivation
Fibrin
Cystic fibrosis
Nanoparticle
Drug delivery
Particle tracking
FRAP
Fluorescence microscopy
Diffusion

ABSTRACT

Diffusion through biological gels is crucial for effective drug delivery using nanoparticles. Here, we demonstrate a new method to measure diffusivity over a large range of length scales – from tens of nanometers to tens of micrometers – using photoactivatable fluorescent nanoparticle probes. We have applied this method to investigate the length-scale dependent mobility of nanoparticles in fibrin gels and in sputum from patients with cystic fibrosis (CF). Nanoparticles composed of poly(lactic-*co*-glycolic acid), with polyethylene glycol coatings to resist bioadhesion, were internally labeled with caged rhodamine to make the particles photoactivatable. We activated particles within a region of sample using brief, targeted exposure to UV light, uncaging the rhodamine and causing the particles in that region to become fluorescent. We imaged the subsequent spatiotemporal evolution in fluorescence intensity and observed the collective particle diffusion over tens of minutes and tens of micrometers. We also performed complementary multiple particle tracking experiments on the same particles, extending significantly the range over which particle motion and its heterogeneity can be observed. In fibrin gels, both methods showed an immobile fraction of particles and a mobile fraction that diffused over all measured length scales. In the CF sputum, particle diffusion was spatially heterogeneous and locally anisotropic but nevertheless typically led to unbounded transport extending tens of micrometers within tens of minutes. These findings provide insight into the mesoscale architecture of these gels and its role in setting their permeability on physiologically relevant length scales, pointing toward strategies for improving nanoparticle drug delivery.

1. Introduction

Nanoparticles hold considerable potential as vehicles for controlled release and targeted delivery of drugs [1]. To achieve therapeutic efficacy, drug-loaded nanoparticles often must diffuse through biological barriers [2–9]. In some instances, the barrier is only a few micrometers thick, as in the case of tear film at the ocular surface [10,11]. In other instances, nanoparticles must penetrate tens of micrometers or more through viscoelastic biological gels or tissue [2,5,9,12]. Measurements of nanoparticle mobility in vitro at these physiologically relevant length scales are valuable for designing particles that exhibit favorable in vivo biodistribution [5–7]. Traditional techniques, such as diffusion chambers, are being replaced by fluorescence microscopy techniques that provide more accurate measurements without perturbing fluid balance or damaging delicate biological specimens, as reviewed elsewhere [3,13].

Here, we introduce a novel fluorescence microscopy strategy for measuring nanoparticle mobility in biological gels at multiple physiologically relevant length scales by employing biodegradable, adhesion-resistant nanoparticles containing a photoactivatable ("caged") fluorescent dye. This functionality enables the photoactivation of particles in a selected region of a sample and subsequent characterization of their

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http://dx.doi.org/10.1016/j.jconrel.2017.05.035
Received 30 January 2017; Received in revised form 19 May 2017; Accepted 30 May 2017
Available online 31 May 2017
0168-3659/

mobility as they spread over tens of micrometers. We call this technique PANDA, for photoactivatable nanoparticle diffusion assay. Extending this range, the motion of individual photoactivated particles can also be examined at high resolution – down to tens of nanometers – using multiple particle tracking (MPT). We have employed PANDA to provide new insight into nanoparticle permeation through biomedically important materials – including fibrin gels and cystic fibrosis (CF) patient sputum – whose adhesive components and mesh architecture pose barriers to drug delivery.

Our approach of using PANDA in conjunction with MPT has a number of advantages over existing fluorescence methods for measuring nanoparticle diffusivity. By itself, MPT is a powerful technique for analyzing many individual particle trajectories, and it has been adopted recently to measure nanomedicine transport in biological materials [14]. However, high-precision MPT requires microscope objectives with large numerical aperture and thus small depths of field [14]. Therefore, particles diffusing in three dimensions can typically be tracked for only several micrometers before diffusing out of focus [15,16]. In heterogeneous materials, diffusivities measured at small length scales might not agree with those measured at larger scales [17,18], and this difference can have important implications in assessing the efficacy of drug delivery strategies. The PANDA method permits direct observation of percolation through biological gels over longer length and time scales but sacrifices spatiotemporal and single-particle resolution. Hence, as we show, MPT and PANDA are complementary and together permit multiscale characterization of diffusion that provides insight into gel microstructure. PANDA also offers advantages over its inverse, fluorescence recovery after photobleaching (FRAP) [19–21], which has been used in drug delivery research for more than two decades [22,23], including for studying nanoparticle diffusion in biological gels [24–26]. Photoactivation generally provides better contrast and signal-to-noise ratio, with signal from the activated fluorophores appearing against a black background [27–29]. Photoactivation can require lower laser dosage than FRAP [27,29], especially in cases of fluorescent nanoparticles, which are often difficult to bleach. Finally, the ability to pattern photoactivated regions with a laser affords PANDA greater control and precision compared to directly microinjecting particles and observing their spread [12].

To date, fluorescence photoactivation has been employed primarily in cell biology [29–32], but one recent paper reported photoactivation experiments investigating molecular diffusion in cellulose-derived gels for microbicide delivery [18]. Our overarching goal here is to extend the application of fluorescence photoactivation and harness it for the study of nanoparticle diffusion through biological gels. One specific aim of this paper is to demonstrate that PANDA can quantify the fluorescent particles' spread over long distances and, importantly, that it is well-suited to observe spatial heterogeneities in diffusion. A second, critical aim of our work is to extend our knowledge of microscale diffusion from MPT measurements into the mesoscale.

As a proof of principle, Section 3.2 presents a comparison of MPT and PANDA measurements on particles diffusing in water. Section 3.3 then describes the application of the methods to fibrin, a protein gel whose microstructure and selective permeability are closely linked to its function, since fibrin forms the scaffolding of blood clots and plays a critical role in hemostasis [33,34]. In these measurements, both MPT and PANDA revealed mobile and immobile populations of particles in fibrin. Finally, in Section 3.4 we examine nanoparticle diffusion in sputum collected from CF patients [35,36]. Secretions coating the airway epithelium pose a major barrier to inhaled nanomedicine for lung diseases such as CF, but particles that can rapidly diffuse through this biological gel may avoid mucociliary clearance and achieve improved distribution, retention, and efficacy in the lungs [36–38]. The airway-surface liquid layer can range from a few micrometers to tens of micrometers thick, depending on location in the lungs and the disease state [39–41]. Employing PANDA, we characterized particle diffusivity in sputum over distances relevant to drug delivery in the lungs, and furthermore, revealed heterogeneities in particle mobility that might be missed with techniques surveying motion over only one length scale.

2. Materials and methods

2.1. Materials

Cholalic acid sodium salt (CHA) and $NVOC_2$-5-carboxy-Q-rhodamine-NHS ester (caged rhodamine-NHS ester) were purchased from Sigma-Aldrich (St. Louis, MO). Poly(lactide-*co*-glycolide(75:25)) amine endcap (PLGA-NH_2), M_n 10–15 kDa was purchased from PolySciTech (West Lafayette, IN). Poly(lactide-*co*-glycolide(67:33))-polyethylene glycol diblock copolymer (PLGA-PEG; 45 kDa PLGA block and 5 kDa PEG block) was custom-synthesized by Jinan Daigang Biomaterial Co., Ltd. (Jinan, China). Human α-thrombin (activity 3059 NIH U/mg) and human fibrinogen (plasminogen depleted, activity 100%), both purified from human plasma, were purchased from Enzyme Research Laboratories (South Bend, IN; catalog numbers HT 1002a and FIB 1, respectively).

2.2. Nanoparticle formulation and characterization

2.2.1. Labeling of PLGA with caged rhodamine

Caged rhodamine-NHS ester and PLGA-NH_2 were conjugated through formation of an amide bond. (Conjugating the dye to polymer in this way, rather than encapsulating the dye in the particles, reduces the likelihood of free dye being released.) Briefly, 90 mg of PLGA-NH_2 was added to 5 mg of caged rhodamine-NHS ester, leading to a slight molar excess of dye compared to PLGA, 1.23:1, and put under vacuum for 1 h. The mixture was then flushed with nitrogen gas, dissolved in 500 μL of anhydrous dichloromethane (DCM), and reacted for 12 h at room temperature under nitrogen gas. Additional DCM was added as needed to facilitate transfer into 10 mL of −20 °C diethyl ether to precipitate the product. The PLGA, now conjugated with the caged rhodamine, was washed twice in cold ether by centrifugation. Excess ether was decanted and the final product, the purified PLGA-caged rhodamine, was placed in a lyophilizer (FreeZone 4.5 Plus; Labconco) for 12 h. The dried product was stored at −20 °C in a shielded container to prevent exposure to incident UV light. It is important to note that the amide bond is formed between the amine end-cap of the PLGA and the succinimidyl (NHS) ester on the rhodamine; that is, the dye itself and not the ortho-nitroveratryloxycarbonyl (NVOC) cage is directly conjugated to PLGA. Thus, when the photolytic reaction occurs upon exposure to UV light, only the caging group is cleaved, and free dye is not released [30,42].

2.2.2. PLGA-PEG particle formulation

Photoactivatable PLGA/PLGA-PEG nanoparticles were prepared using the emulsion solvent evaporation method [43]. Briefly, a 40 mg mixture (19:1 by mass) of PLGA45k-b-PEG5k and PLGA-caged rhodamine was dissolved in 400 μL of DCM, making a 100 mg/mL solution. This solution was injected into an ice-cooled vial containing 5 mL of 0.5% CHA aqueous solution, and sonicated at 30% amplitude for 2 min using a 130 W probe sonicator (Sonics & Materials, Newtown, CT) to form an oil-in-water emulsion of the organic phase (polymer and DCM) in the aqueous phase, stabilized by the CHA surfactant. The emulsion was immediately added to 35 mL of 0.5% CHA solution and stirred at 600 rpm for at least 3 h to allow for complete particle hardening. During particle formation, the hydrophilic PEG partitioned to the surface of the emulsion drop, while the relatively hydrophobic PLGA remained in the interior, resulting in particles with a dense PEG coating of approximately 20 PEG chains per 100 nm^2 [43,44]. The final particle suspension was filtered through a 5 μm and then 0.45 μm syringe filter, then the particles were collected and washed three times via centrifugation at 20,000 g for 25 min. The final suspension in DI water was stored at 4 °C. Particles were typically used within 5 days of synthesis,