Journal of Controlled Release 231 (2016) 50–59

Contents lists available at ScienceDirect

Journal of Controlled Release

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

Tracking and quantifying polymer therapeutic distribution on a cellular level using 3D dSTORM

Jonathan M. Hartley [a], Rui Zhang [b], Manasa Gudheti [c,d], Jiyuan Yang [b], Jindřich Kopeček [a,b,*]

[a] Department of Bioengineering, University of Utah, 20 S. 2030 E., Rm. 108, Salt Lake City, UT 84112, USA
[b] Department of Pharmaceutics and Pharmaceutical Chemistry, University of Utah, 20 S. 2030 E., Rm. 205, Salt Lake City, UT 84112, USA
[c] Department of Biology, University of Utah, 257S 1400 E, Salt Lake City, UT 84112, USA
[d] Bruker Nano Surfaces, 630 Komas Drive, Salt Lake City, UT 84108, USA

ARTICLE INFO

Article history:
Received 11 November 2015
Received in revised form 27 January 2016
Accepted 3 February 2016
Available online 16 February 2016

Keywords:
Drug delivery
N-(2-hydroxypropyl)methacrylamide (HPMA)
dSTORM
Super resolution

ABSTRACT

We used a single-molecule localization technique called direct stochastic optical reconstruction microscopy (dSTORM) to quantify both colocalization and spatial distribution on a cellular level for two conceptually different *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer conjugates. Microscopy images were acquired of entire cells with resolutions as high as 25 nm revealing the nanoscale distribution of the fluorescently labeled therapeutic components. Drug-free macromolecular therapeutics consisting of two self-assembling nanoconjugates showed slight increase in nanoclusters on the cell surface with time. Additionally, dSTORM provided high resolution images of the nanoscale organization of the self-assembling conjugates at the interface between two cells. A conjugate designed for treating ovarian cancer showed that the model drug (Cy3) and polymer bound to Cy5 were colocalized at an early time point before the model drug was enzymatically cleaved from the polymer. Using spatial descriptive statistics it was found that the drug was randomly distributed after 24 h while the polymer bound dye remained in clusters. Four different fluorescent dyes were used and two different therapeutic systems were tested to demonstrate the versatility and possible general applicability of dSTORM for use in studying drug delivery systems.

1. Introduction

Nano drug delivery systems often involve complex assemblies of various materials including drugs, synthetic polymers [1,2], lipids [3], peptides [4], carbohydrates [5] or oligonucleotides [6]. The system complexity is required to overcome barriers between the point of injection and the target site [7]. Therapeutics must evade immunogenic triggers, cross cellular membranes, cross nuclear pore complex, etc. to reach their target. Often nano drug delivery systems need to undergo chemical reactions or assembly steps to properly cross a barrier or exert their therapeutic effect [8]. To properly study the mechanisms of nano drug delivery systems, new tools are needed to visualize and quantify their effects on individual cells and track their distribution at a cellular level [9]. We used a single-molecule point localization technique called direct stochastic optical reconstruction microscopy (dSTORM) to visualize and quantify cellular distribution of two conceptually different HPMA copolymer therapeutic systems to demonstrate the tool's general applicability in drug delivery.

Discoveries related to controlling fluorescence of proteins and synthetic dyes fueled advances in optical imaging where individual fluorescent molecules can be localized [10–13]. Some single molecule localization approaches include photoactivated localization microscopy (PALM) [10], STORM [13], dSTORM [12], fluorescence PALM [14], bleaching/blinking-assisted localization microscopy (BaLM) [15], ground-state depletion microscopy followed by individual molecule return (GDSDIM) [16], and generalized single-molecule high-resolution imaging with photobleaching (gSHRiMP) [17]. In dSTORM imaging, a single fluorescent dye is used along with an appropriate imaging buffer that has thiol-containing compounds (such as mercaptoethylamine) in solution [12]. The thiol groups react with the dye upon laser irradiation to put the dye into a metastable dark state thereby controlling its fluorescence [18]. Controlling dye fluorescence enables the activation of sparse subsets of fluorophores that can be individually localized because the fluorescence signal from each molecule is spaced far enough apart that signals do not overlap. Since the fluorescent signals do not overlap the centroid of the fluorescent spot can be precisely localized by fitting the signal to a Gaussian point spread function. Each localized molecule is fit to the point spread function and a centroid of the signal is easily obtained. The uncertainty of the centroid depends upon the number of photons collected and how well the signal fits the point spread function. The process of activating and localizating sparse subsets of molecules in individual frames is repeated thousands of times after which a super-resolution image is constructed from the fitted localizations of all the molecules in every frame. A good fit and a

* Corresponding author at: Department of Pharmaceutics and Pharmaceutical Chemistry, University of Utah, 20 S. 2030 E., Rm. 205, Salt Lake City, UT 84112, USA.
E-mail address: jindrich.kopecek@utah.edu (J. Kopeček).

http://dx.doi.org/10.1016/j.jconrel.2016.02.005
0168-3659/

sufficient number of photons can result in images with resolutions as high as 10 nm. Image resolution is enhanced by an order of magnitude over traditional optical microscopy methods such as confocal microscopy and total internal reflection fluorescence microscopy (TIRFM). These discoveries and advances have resulted in elucidation of cellular structures such as Tar clustering in *Escherichia coli* [19], MreB helical organization, an actin analog [20], and the hemispherical clathrin coat [21]. Many commercially available fluorescent probes can be used in dSTORM imaging [22]. Because dSTORM localizes individual molecules, the resulting image contains coordinate data, photon count, and single molecule resolution precision.

The coordinate data can then be used to quantitatively study drug delivery mechanisms using spatial descriptive statistical techniques such as pair-correlation analysis [23,24]. Spatial descriptive statistical techniques allow researchers to quantify the distribution of drugs, delivery vehicles, or proteins (i.e. cluster size, packing density) [23,25]. For example pair-correlation functions can be determined from an image and give rise to correlation lengths, which are related to the size of structures in the image [26]. Correlation functions can also be used to determine other important biophysical parameters in complex systems [26]. Recently, we used pair-correlation analysis to calculate CD20 protein cluster size and estimate the density of CD20 in the clusters from 2D dSTORM images [27]. Traditional optical imaging techniques (confocal, TIRFM) cannot produce the resolution needed to observe and calculate the size of nanoscale clusters, nor can they provide individual coordinate data for each molecule, thereby precluding use of statistical methods to extract valuable biophysical data.

The two *N*-(2-hydroxypropyl)methacrylamide (HPMA) therapeutic systems we imaged differ in the function of the HPMA copolymer and in the cellular target. The drug-free macromolecular therapeutic system relies on the biorecognition at the cell surface of oligonucleotides or coiled-coil peptides conjugated to a HPMA polymer and an anti-CD20 Fab′ fragment to physically crosslink CD20 and induce apoptosis [6,28,29]. In the drug-free system, the HPMA copolymer acts as a physical crosslinker between hybridized pairs of complementary oligonucleotides or peptides and CD20 on the cell surface rather than a carrier of a cytotoxic drug. The biorecognition of the oligonucleotides or coiled-coil peptides resulted in crosslinked CD20 on the surface of the cell, thereby initiating apoptosis. One of the nanoconjugates is an anti-CD20 Fab′ fragment from the monoclonal antibody 1F5 covalently attached to a 25 base pair oligonucleotide (Fig. 1). Multiple copies of the complementary morpholino are covalently attached to a HPMA copolymer, forming a hybrid graft copolymer (Fig. 1). This conjugate system has shown efficacy in Burkitt's lymphoma mouse models and has recently shown promising results in vitro against patient cells of mantle cell lymphoma [30] and chronic lymphocytic leukemia (CLL) [31] patient samples. Currently, the oligonucleotides (MORF1/MORF2) are used in our lab due to their superior binding affinity and apoptosis inducting in vitro and in vivo compared to coiled-coil peptides (CCE/CCK) [6,32].

The HPMA conjugate designed to treat ovarian cancer is a diblock copolymer with a GFLG peptide sequence linking the two blocks, and the model drug (Cy3) was tethered to the polymer via an enzyme degradable peptide sequence, GFLG (Fig. 2) [33]. The GFLG peptide sequence was introduced into the copolymer backbone so that higher molecular weight polymer conjugates (~80–100 kDa) could be synthesized [34,35], otherwise only lower molecular weight polymers (~40–50 kDa), which have lower circulation times and therefore lower accumulation in solid tumors, could be used to ensure excretion by the kidneys. For the conjugate to deliver the model drug the cell must internalize it, and then the "drug" (Cy3) is cleaved enzymatically from the polymer. The polymer functions as a carrier for the cytotoxic drug or agent to prevent its release while in circulation to prevent adverse effects from the drug cytotoxicity and to increase circulation time of the polymer drug conjugate to enhance accumulation inside the tumor.

The principal aims of this paper were to quantify nanoconjugate localization and to show that dSTORM is versatile in that it can be applied to functionally different therapeutic systems. We synthesized drug-free nanoconjugates representing both biorecognition strategies of hybridization and coiled-coil formation, and we also synthesized a conjugate for ovarian cancer with a cleavable diblock copolymer backbone. Components of the conjugates were labeled with one of five different fluorophores (Cy5, Alexa Fluor 647, Cy3B, Cy3, and FITC) four of which are dSTORM compatible. We used 3D dSTORM to track the labeled drug-free nanoconjugates and diblock conjugate in human Burkitt's lymphoma cells and human ovarian cancer cells respectively. The localization of the drug-free conjugates was imaged at 2 h and 6 h while the distribution of the diblock components in ovarian cancer cells was imaged at 4 h and 24 h.

2. Materials and methods

2.1. Materials

The solvents dichloromethane (DCM), methanol, diethyl ether, acetone and dimethylformamide (DMF) were purchased from Fisher Scientific (Pittsburgh, PA). Cysteamine, glucose oxidase type seven from *Aspergillus niger*, catalase from bovine liver, piperidine, trifluoroacetic acid (TFA), triisopropylsilane (TIS), and diisopropylethylamine (DIPEA) were purchased from Sigma-Aldrich (St. Louis, MO). Amino acids and 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU) were purchased from AAPPTEC (Louisville, KY). The fluorescent probes Cy3-NHS ester, Cy5-amine, and Cy5-NHS ester were purchased from Lumiprobe (Hallandale Beach, FL). Alexa Fluor 647 NHS ester was purchased from Life Technologies (Carlsbad, CA). The Cy3B-NHS ester was purchased from GE Healthcare Life Sciences (Pittsburgh, PA). The heterobifunctional linker succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) was purchased from Highfine Biotech (Suzhou, China). The initiators 2,2′-azobis[2-(2-imidazolin-2-yl)-propane]dihydrochloride (VA-044), 2,2-azobis(2,4-dimethyl valeronitrile) (V-65), and 4,4′-azobis(4-cyanopentanoic acid) (V-501) were purchased from Wako Chemicals (Richmond, VA). The monomer *N*-(3-aminopropyl)methacrylamide hydrochloride (APMA) was purchased from Polysciences, Inc. (Warrington, PA). Other monomers and chain transfer agents were synthesized as described previously: HPMA [36], 3-(*N*-methacryloylglycylglycyl) thiazolidine-2-thione (MA-GG-TT) [37], 2-(*N*-methacryloylglycylphenyl alanylleucylglycine)-*N*′-Boc-ethylenediamine (MA-GFLG-NH-Boc) [38], methacryloylated fluorescein (MA-FITC), and RAFT chain transfer agents, 4-cyanopentanoic acid dithiobenzoate (CPDB) [39], and peptide2CTA (N^{α},N^{ε}-bis(4-cyano-4-(phenylcarbonothioylthio)pentanoylglycylphenyl alanylleucylglycyl)lysine) [35]. Tris/HCL, 1 M solution, pH 8.0 ultrapure was purchased from USB Corporation (Cleveland, OH). Glass bottom microwell dishes used for dSTORM imaging were purchased from MatTek (Ashland, MA). The reducing agent tris(2-carboxyethyl)phosphine hydrochloride (TCEP) was purchased from Gold Biotechnology (St. Louis, MO). Oligonucleotides (MORF1-NH_2 and MORF2-SH) were purchased from Gene Tools (Philomath, OR).

2.2. Coiled-coil peptide synthesis

The peptides, CCE and CCK, [40] were synthesized using solid-phase peptide synthesis with an Fmoc/tBu strategy on 2-chlorotrityl chloride resin. A spacer consisting of tyrosine and two glycines was added to the N terminus, and then the peptide was finally modified with either Cys (for conjugation to HPMA) or SMCC. The peptides were cleaved from the resin using 95% TFA, 2.5% TIS, and 2.5% H_2O (EDT was added for the peptide modified with Cys). The beads were removed and the solution condensed before precipitating the peptides in cold diethyl ether. Peptide purification was performed using reverse-phase high pressure liquid chromatography (RP-HPLC) on a semi-preparative Zorbax