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Stimulated Raman scattering of polymer nanoparticles for multiplexed live-cell imaging

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Abstract

A novel nanoparticle-based imaging strategy is introduced that couples biocompatible organic nanoparticles and stimulated Raman scattering (SRS) microscopy. Polymer nanoparticles with vibrational labels incorporated were readily prepared for multicolor SRS imaging with excellent photo-stability. The Raman-active polymer dots are nontoxic, rapidly enter various cell types, and are applied in multiplexed cell-type sorting.

Molecules and nanomaterials that can be readily imaged have found great utility as contrast agents in biological and medical research¹⁻³. Recently, metal nanoparticles and quantum dots (Q-dots) have become an attractive platform for bioimaging⁴⁻⁵. Q-dots exhibit narrow light emission and high quantum yields that enable multiplexing, and metal nanoparticles offer precise shape tunability. However, these systems have been shown to be less than ideal in biological applications due to inherent limitations, including cytotoxicity from metal ion leaching, elaborate surface processing to maintain stability in fluctuating environments, and small scale syntheses with batch variability⁶⁻⁸. Alternatively, carbon-based materials such as graphene⁹, carbon nanotubes¹⁰, and nanodiamonds¹¹ have been developed as contrast agents in live-cell imaging, but they require precise control of chemical composition for reproducibility¹².

Different from metal- or carbon-based materials, metal-free polymer nanoparticles have great potential in bioimaging and theranostics because of their modular chemical functionality¹³⁻¹⁴. Unfortunately, most polymeric nanoparticles rely on fluorescence reporting, and hence are limited by the intrinsic photophysics of fluorophores¹⁵, including self-quenching, broad emission, and photobleaching of organic dyes. Further compromising widespread use of polymer-based materials is the synthesis of complex architectures, such as bottle-brushes, star-shaped nanoparticles, and hollow nanomaterials¹³. Therefore, it is imperative to explore beyond conventional imaging agents and techniques. Developing a biocompatible platform of all-polymer nanoparticles that can be readily synthesized and optically imaged in live cells with desirable contrast mechanism could significantly impact biotechnological and diagnostic applications.

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Stimulated Raman scattering (SRS) is an emerging non-linear vibrational imaging technique that can overcome many limitations of fluorescence imaging and other metal-based nanoparticle imaging techniques¹⁶⁻¹⁹. Compared to conventional spontaneous Raman imaging, SRS provides 10^8 enhancement in excitation efficiency and over 1000-fold improvement in imaging speed through stimulated emission of vibrational transitions by an additional Stokes laser beam²⁰⁻²¹. SRS is intrinsically free from photobleaching and blinking, and does not rely on metallic nanostructures for localized plasmonic enhancement such as in surface enhanced Raman scattering⁸. In addition, the use of picosecond near-infrared lasers permits narrow-band detection of sharp vibrational peaks and deep tissue penetration with reduced phototoxicity. Vibrational labelling has been recently coupled with SRS microscopy as a powerful technique for bioorthogonal chemical imaging of small biomolecules in live cells and tissues²²⁻²⁷. Thus far, SRS has been limited to small molecules probes, which must be custom-designed to be water soluble, non-cytotoxic, and detectable under high dilution for live-cell imaging.

Functional synthetic latex nanoparticles represent a modular platform to generalize SRS imaging probes. Miniemulsion polymerization is a facile synthetic method that has been shown to reproducibly yield large-scale quantities of polymer nanoparticles with narrow size distribution²⁸. Here, we show how the chemistry of the prototype nanoparticle can be exploited to develop a new family of latexes for multiplexed live-cell imaging by SRS (Figure 1). Formation of the latex is enabled by a functional surfactant that is covalently incorporated through the hydrophobic styrene moiety, while displaying the hydrophilic head group on the surface of the spherical nanoparticle²⁹. The hydrophilic group is composed of a soft, highly delocalized trisaminocyclopropenium (TAC) cation that affords a positive surface charge and resistance to flocculation across a wide range of pH values³⁰. Importantly, the hydrophobic polymer core is readily obtained through the radical polymerization of styrene and its derivatives. Introducing characteristic chemical bonds as vibrational tags onto the styrene monomer allows high labelling efficiency and enables SRS imaging with high sensitivity and specificity. We refer to this new family of polymer latexes as Raman-active polymer dots.

A key attractive feature of the Raman-active polymer dots is their facile synthesis. The parent oil-in-water emulsion is obtained by adding a 95:5, w:w ratio of styrene monomer to TAC surfactant in water. A stable latex is obtained by radical polymerization using V-50, a thermally active initiator, and heating to 70°C, followed by a 24-h dialysis to remove unreacted monomer. The use of styrene provides a rich palette to decorate the aromatic unit with various vibrational tags for Raman imaging, without disturbing the size distribution of the oil-in-water emulsion. Using styrene derivatives with tiny vibrational labels to dope the emulsion furnished a library of Raman-active polymer dots (Figure 2). The uniform dot size can be varied from 30-100 nm by changing the ratio of monomer to surfactant (Table S1, Figure S1-S4), with nearly 10^5 vibrational labels incorporated in each polymer dot.

Alkyne, nitrile, and carbon-deuterium bonds (C-D) were chosen as bioorthogonal vibrational tags since they all exhibit characteristic and mutually resolvable peaks with narrow bandwidth (< 2 nm) in the Raman region where cells possess minimal endogenous vibrations (referred to as the cell Raman-silent region, 1740-2800 cm^{-1} , Figure 2 and Figure

S5)³¹. Both the alkyne and nitrile bonds were conjugated with the phenyl ring of styrene for enhanced Raman cross sections³¹. A trimethylsilane-protecting group was employed to further improve the alkyne signal strength while maintaining its frequency (2163 cm⁻¹, CC-dot) well separated from that of the nitrile (2232 cm⁻¹, CN-dot). Additionally, use of d₅-styrene fully separates the frequency (2293 cm⁻¹, CD-dot) from the nitrile vibration, compared to d₈-styrene that exhibits a broad spectrum due to the backbone C-D vibrations³².

We next studied the cellular behavior of Raman-active polymer dots as contrast agents in live-cell imaging. The latexes were synthesized without the use of any organic solvents or extra surface modification enabling their rapid application into cells. Live HeLa cells were separately incubated with CC-dots, CN-dots, or CD-dots and we found that all Raman-active polymer dots efficiently entered the cells within 2 hours and accumulated mainly in vesicular structures near the cell nucleus (Figure 3), which is confirmed by spontaneous Raman measurements (Figure S6). Imaging at the characteristic frequencies of alkyne (2163 cm⁻¹), nitrile (2232 cm⁻¹), and C-D (2293 cm⁻¹), cells incubated with the corresponding Raman-active nanoparticles show exclusive SRS signal with high signal to noise ratio (S/N>5, at 30 μs time constant) in the specific on-resonance channel (the colors in all images are pseudo-colors assigned to each frequency). Thus, the three Raman-active polymer nanoparticles were found to be spectrally orthogonal to each other in live cells with negligible cross-talk, allowing high fidelity multiplexed imaging without the need of spectral unmixing the signal output.

To determine the intracellular locations of polymer nanoparticles, the endosomes were co-stained in live HeLa cells with transferrin Texas Red conjugate. The correlative SRS and fluorescence imaging revealed high spatial co-localization between the Raman-active nanoparticle puncta in the SRS channel and the endosome marker in the fluorescence channel, suggesting the cellular entry of nanoparticles through endocytosis³³ (Figure 3d). Incubating HeLa cells with nanoparticles at 4°C fully suppressed their cellular entry, further supporting the endocytic mechanism of entry³³ (Figure S7).

Time-dependent cellular entry kinetics were also characterized for all three Raman-active polymer dots. In as short as 1 hour, observable quantities of nanoparticles were found inside the cells, indicating fast entry kinetics³⁴, likely due to the positively-charged nanoparticle surface. This is in agreement with other studies showing positively-charged surfaces and nanoparticle sizes of ~50 nm are ideal for rapid cellular uptake³⁴. After 4 hours of incubation, significant amounts of nanoparticles are enriched in the endosomes surrounding the cell nucleus (Figure S8). Thus, the three Raman-active polymer dots were found to behave similarly with rapid entry via endocytosis within an hour, highlighting the fact that doping the styrene latexes with different vibrational labels does not introduce variability in their interaction with live cells.

Unlike fluorescent polymer nanoparticles that require electronic excitation, Raman scattering is based on chemical bond vibration, which is intrinsically free from photobleaching, blinking, and self-quenching. Thus, Raman-active polymer nanoparticles were expected to exhibit excellent photo-stability for long-term imaging. Indeed, by continuously imaging the nanoparticles in live HeLa cells for 100 frames (2.7 second per

frame), we found little reduction in intensity (Figure 4a) and that the strong signal is stable (without bleaching or blinking) after nearly 300-second of laser illumination, as shown quantitatively by the steady intensity time traces (Figure 4b). Compared to other imaging contrast agents which typically show fluorescence intermittency or rapid decay, Raman-active polymer dots display high photo-stability under SRS microscopy and are exceptionally well suited for tracking applications in live cells for long periods of time, as shown in the video (Movie S1).

To demonstrate the broad applicability of Raman-active polymer dots in live-cell imaging, we tested their cellular entry capacity and biocompatibility in five cell lines. In addition to HeLa, two other cancerous cell lines were evaluated (human breast cancer cells MCF-7 and human fibrosarcoma cells HT1080), as well as two non-cancerous cell lines (monkey kidney fibroblast-like cells COS-7 and mouse embryonic fibroblasts MEF). Incubated with the alkyne labelled polymer dots, all four additional cell lines were efficiently labelled within 2 hours with strong accumulation of the Raman-active dots inside the cells (Figure S9). Furthermore, cytotoxicity assays showed all five cell lines (COS-7, MEF, MCF-7, HT1080 and HeLa) have greater than 85% (some even more than 95%) viability with all three Raman-active dots after 48-hour culture (Figure S10). The biocompatible chemical composition of our organic nanoparticles should be responsible for such negligible cytotoxicity. By contrast, Q-dots without surface coating display acute toxicity at similar loadings³⁵. These data demonstrate that Raman-active polymer dots exhibit minimal toxicity in all cell lines tested and would be widely applicable as imaging agents across a broad range of cells.

The ability to quickly identify cell type in a multi-component system will allow fast readout of information in many applications such as cell screening or monitoring long-term spatial migration. As the three-color Raman-active polymer dots exhibit high detection orthogonality and biocompatibility across multiple cell lines, we employed them for multiplexed live-cell sorting in co-culture by SRS microscopy. Each cell type was “color-coded” by incubation with one specific Raman-active polymer dot (alkyne for COS-7, nitrile for MEF, and deuterium for HeLa cells), after which the three cell types were mixed and cultured together.

After co-culture of 4 and 24 hours, cells could be unambiguously identified by their polymer dot labelling (Figure 5). In both cases, cells were indistinguishable by simple morphology inspection or by label-free SRS imaging of the lipid CH₂ vibration (2845 cm⁻¹). However, by imaging through the unique frequencies of alkyne, nitrile, and C-D, every cell in the frame can be rapidly and singularly identified by the color-coding of the Raman-active polymer dots. By merging all three channels together with the lipid CH₂ image, not only the identity of the cell type, but also the spatial location of each cell in a co-culture can be clearly mapped out. This demonstrates multi-color Raman-active polymer dots as promising live-cell multiplexed imaging agents.

We have developed a new strategy to image organic polymeric nanoparticles via stimulated Raman scattering that is free from the use of fluorescent dyes or metals. Characteristic chemical bonds of minimal size and orthogonal vibrational modes with narrow bandwidth

were rationally designed and incorporated into organic polymer nanoparticles in a robust one-step synthesis. The nanoparticles were shown to have rapid cellular entry kinetics and high biocompatibility across a broad range of cell lines. We have achieved near-infrared multi-color SRS imaging of these materials in live cells demonstrating high sensitivity, specificity, and photo-stability. We further applied this technology in multiplexed live-cell sorting with unambiguous identification of cell types in co-culture with fast readout. The multiplexing capability can be further expanded by precisely tuning the vibrational frequencies of the alkyne and nitrile through isotope editing³⁶, and efforts to target these nanoparticles towards specific cell types are ongoing. Addressing the previous issues of cytotoxicity, photo-stability and synthetic robustness for nanoparticle-based imaging reagents, we anticipate that multiplexed Raman-active polymer dots coupled with SRS microscopy will be an important technology in nanoparticle-based live-cell imaging for biotechnology and theranostic applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Notes and references

1. Salata OV. Applications of nanoparticles in biology and medicine. *J Nanobiotechnol.* 2004; 2:3–3.
2. Welch MJ, Hawker CJ, Wooley KL. The Advantages of Nanoparticles for PET. *J Nucl Med.* 2009; 50(11):1743–1746. [PubMed: 19837751]
3. Wolfbeis OS. An overview of nanoparticles commonly used in fluorescent bioimaging. *Chem Soc Rev.* 2015; 44(14):4743–4768. [PubMed: 25620543]
4. Michalet X, Pinaud FF, Bentolila LA, Tsay JM, Doose S, Li JJ, Sundaresan G, Wu AM, Gambhir SS, Weiss S. Quantum Dots for Live Cells, in Vivo Imaging, and Diagnostics. *Science.* 2005; 538:538–545.
5. Lane LA, Qian X, Nie S. SERS Nanoparticles in Medicine: From Label-Free Detection to Spectroscopic Tagging. *Chem Rev.* 2015; 115(19):10489–10529. [PubMed: 26313254]
6. Zhu ZJ, Yeh YC, Tang R, Yan B, Tamayo J, Vachet RW, Rotello VM. Stability of quantum dots in live cells. *Nat Chem.* 2011; 3:963–968. [PubMed: 22109277]
7. Bogart LK, Pourroy G, Murphy CJ, Puentes V, Pellegrino T, Rosenblum D, Peer D, Lévy R. Nanoparticles for Imaging , Sensing , and Therapeutic Intervention. *ACS Nano.* 2014; 8(4):3107–3122. [PubMed: 24641589]
8. Song ZL, Chen Z, Bian X, Zhou LY, Ding D, Liang H, Zou YX, Wang SS, Chen L, Yang C, Zhang XB, Tan W. Alkyne-Functionalized Superstable Graphitic Silver Nanoparticles for Raman Imaging. *J Am Chem Soc.* 2014; 136(39):13558–13561. [PubMed: 25233109]
9. Sun X, Liu Z, Welsher K, Robinson JT, Goodwin A, Zaric S, Dai H. Nano-Graphene Oxide for Cellular Imaging and Drug Delivery. *Nano Res.* 2008; 1(3):203–212. [PubMed: 20216934]
10. Liu Z, Li X, Tabakman SM, Jiang K, Fan S, Dai H. Multiplexed Multicolor Raman Imaging of Live Cells with Isotopically Modified Single Walled Carbon Nanotubes. *J Am Chem Soc.* 2008; 130(41):13540–13541. [PubMed: 18803379]

11. Pope I, Payne L, Zorinants G, Thomas E, Williams O, Watson P, Langbein W, Borri P. Coherent anti-Stokes Raman scattering microscopy of single nanodiamonds. *Nat Nanotech.* 2014; 9:940–946.
12. Hong G, Diao S, Antaris AL, Dai H. Carbon Nanomaterials for Biological Imaging and Nanomedicinal Therapy. *Chem Rev.* 2015; 115(19):10816–10906. [PubMed: 25997028]
13. Elsabahy M, Heo GS, Lim SM, Sun G, Wooley KL. Polymeric Nanostructures for Imaging and Therapy. *Chem Rev.* 2015; 115:10967–11011. [PubMed: 26463640]
14. Wu C, Chiu DT. Highly Fluorescent Semiconducting Polymer Dots for Biology and Medicine. *Angew Chem Int Ed.* 2013; 52(11):3086–3109.
15. Reisch A, Klymchenko AS. Fluorescent Polymer Nanoparticles Based on Dyes: Seeking Brighter Tools for Bioimaging. *Small.* 2016; 12(15):1968–92. [PubMed: 26901678]
16. Freudinger CW, Min W, Saar BG, Lu S, Holtom GR, He C, Tsai JC, Kang JX, Xie XS. Label-Free Biomedical Imaging with High Sensitivity by Stimulated Raman Scattering Microscopy. *Science.* 2008; 1857:1857–1861.
17. Cheng JX, Xie XS. Vibrational spectroscopic imaging of living systems: An emerging platform for biology and medicine. *Science.* 2015; 350(6264):aaa8870. [PubMed: 26612955]
18. Prince RC, Frontiera RR, Potma EO. Stimulated Raman Scattering: From Bulk to Nano. *Chem Rev.* 2016 Article ASAP.
19. Liao CS, Cheng JX. In Situ and In Vivo Molecular Analysis by Coherent Raman Scattering Microscopy. *Annu Rev Anal Chem.* 2016; 9(1):69–93.
20. Min W, Freudiger CW, Lu S, Xie XS. Coherent Nonlinear Optical Imaging: Beyond Fluorescence Microscopy. *Annu Rev Phys Chem.* 2011; 62:507–530. [PubMed: 21453061]
21. Krafft C, Schie IW, Meyer T, Schmitt M, Popp J. Developments in spontaneous and coherent Raman scattering microscopic imaging for biomedical applications. *Chem Soc Rev.* 2016; 45(7):1819–1849. [PubMed: 26497570]
22. Wei L, Hu F, Chen Z, Shen Y, Zhang L, Min W. Live-Cell Bioorthogonal Chemical Imaging: Stimulated Raman Scattering Microscopy of Vibrational Probes. *Acc Chem Res.* 2016; 49:1494–1502. [PubMed: 27486796]
23. Hong S, Chen T, Zhu Y, Li A, Huang Y, Chen X. Live-cell stimulated Raman scattering imaging of alkyne-tagged biomolecules. *Angew Chem Int Ed.* 2014; 53(23):5827–5831.
24. Lee HJ, Zhang W, Zhang D, Yang Y, Liu B, Barker EL, Buhman KK, Slipchenko LV, Dai M, Cheng JX. Cholesterol Storage in Live Cells and *C. elegans* by Stimulated Raman Scattering Imaging of Phenyl-Diyne Cholesterol. *Sci Rep.* 2015; 5:7930. [PubMed: 25608867]
25. Alfonso-García A, Pfisterer SG, Riezman H, Ikonen E, Potma EO. D38-cholesterol as a Raman active probe for imaging intracellular cholesterol storage. *J Biomed Opt.* 2015; 21(6):061003–061003.
26. Hu F, Lamprecht MR, Wei L, Morrison B, Min W. Bioorthogonal chemical imaging of metabolic activities in live mammalian hippocampal tissues with stimulated Raman scattering. *Sci Rep.* 2016; 6:39660. [PubMed: 28000773]
27. Hu F, Chen Z, Zhang L, Shen Y, Wei L, Min W. Vibrational Imaging of Glucose Uptake Activity in Live Cells and Tissues by Stimulated Raman Scattering. *Angew Chem Int Ed.* 2015; 54(34):9821–9825.
28. Choi YT, El-Aasser MS, Sudol ED, Vanderhoff JW. Polymerization of styrene miniemulsions. *J Polym Sci Part A Polym Chem.* 1985; 23(12):2973–2987.
29. Jiang Y, Freyer JL, Cotanda P, Brucks SD, Killops KL, Bandar JS, Torsitano C, Balsara NP, Lambert TH, Campos LM. The evolution of cyclopropenium ions into functional polyelectrolytes. *Nat Commun.* 2015; 6:5950. [PubMed: 25575214]
30. Killops KL, Brucks SD, Rutkowski KL, Freyer JL, Jiang Y, Valdes ER, Campos LM. Synthesis of Robust Surface-Charged Nanoparticles Based on Cyclopropenium Ions. *Macromolecules.* 2015; 48(8):2519–2525.
31. Yamakoshi H, Dodo K, Palonpon A, Ando J, Fujita K, Kawata S, Sodeoka M. Alkyne-Tag Raman Imaging for Visualization of Mobile Small Molecules in Live Cells. *J Am Chem Soc.* 2012; 134(51):20681–20689. [PubMed: 23198907]

32. Condirston DA, Laposa JD. Vibrational spectra of styrene-H8, -D3, -D5, and -D8. *J Mol Spectrosc.* 1976; 63(3):466–477.
33. Jaiswal JK, Mattoussi H, Mauro JM, Simon SM. Long-term multiple color imaging of live cells using quantum dot bioconjugates. *Nat Biotechnol.* 2003; 21:47–51. [PubMed: 12459736]
34. Raffaele F, Monica L, Francesca F, Paolo B, Katia P, Fabio F, Cinzia B, Mario S, Maurizio DI, Massimo M, Davide M, Paolo U. Integrated multiplatform method for in vitro quantitative assessment of cellular uptake for fluorescent polymer nanoparticles. *Nanotechnology.* 2014; 25(4): 045102. [PubMed: 24398665]
35. Derfus AM, Chan WCW, Bhatia SN. Probing the Cytotoxicity of Semiconductor Quantum Dots. *Nano Lett.* 2004; 4(1):11–18. [PubMed: 28890669]
36. Chen Z, Paley DW, Wei L, Weisman AL, Friesner RA, Nuckolls C, Min W. Multicolor Live-Cell Chemical Imaging by Isotopically Edited Alkyne Vibrational Palette. *J Am Chem Soc.* 2014; 136(22):8027–8033. [PubMed: 24849912]

Fig 1.
Schematic formulation of the latex: Miniemulsion polymerization of Raman-active polymer dots.

Fig 2.

Spontaneous Raman spectra and structure of three distinct Raman-active polymer dots. (a) Spontaneous Raman spectra of nanoparticles with orthogonal vibrational modes of alkyne, nitrile, and carbon-deuterium bonds in the cell Raman-silent region (from 1740 to 2800 cm^{-1}). The intensity is scaled based on the peaks in the Raman-silent region. (b) Schematic structure of three Raman-active polymer dots with the core comprising styrene and styrenic derivatives and the surface coated with trisaminocyclopropenium (TAC) groups.

Fig 3.

SRS imaging of Raman-active polymer dots entry in live HeLa cells. HeLa cells are incubated with the (a) CC-dot (b) CN-dot, and (c) CD-dot. Each Raman-active polymer dot shows distinct frequency (CC-dot = 2163 cm⁻¹, CN-dot = 2232 cm⁻¹, and CD-dot = 2293 cm⁻¹) without cross-talk in cells. The images at 2845 cm⁻¹ are lipid CH₂ channels showing cell morphology. (d) CC-dots highly co-localize with endosome marker in live HeLa cells, suggesting endocytic entry.

Fig 4. High photo-stability of Raman-active polymer dots in live cells. (a) SRS imaging of alkyne labelled polymer dot in HeLa cells. Representative frames (1, 20, 50 and 100) are shown to display little change in intensity. (b) Intensity quantification of SRS images of Raman-active polymer dots in HeLa cells. Steady intensity traces of both the whole frame and 3 selected regions (white boxes) demonstrate high photo-stability of Raman-active polymer dots after 100 frames.

Fig 5. Multiplexed live-cell sorting in co-culture of three cell lines. Each cell line is color-coded with specific Raman-active polymer dots (alkyne for COS-7, nitrile for MEF and C-D for HeLa) before being mixed in the co-culture. Cell types are unambiguously identified by 3-color polymer dots with multiplexed SRS imaging after 4 hours (a) and 24 hours (b).