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Halide Perovskite Nanocrystals for Multiphoton Applications

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Halide perovskite nanocrystals (NCs) are a unique class of NCs with novel properties distinct from traditional semiconductor NCs. These exceptional properties of defect tolerance, large absorption coefficients, high brightness, and narrow emission linewidths stem from their atypical band structure. Their facile synthesis and broad colour tunability have garnered widespread interests for applications in light emitting devices and lasers. One fledging niche area is the field of multiphoton excited emission where their giant nonlinear optical action cross-sections are highly favorable for imaging applications. This Frontier article examines the state-of-the-art in perovskite NCs for multiphoton applications from the materials science and physics perspectives that include their synthesis and non-linear optical characterization. In particular, opportunities and challenges for these exceptional perovskite NCs as potential fluorescence labels for multiphoton deep tissue microscopy are highlighted.

Introduction

Multiphoton excitation (MPE) is a nonlinear optical process that involves the simultaneous absorption of two or more photons to promote a fluorophore from its ground state to an excited state. This phenomenon has spawned many important applications spanning the fields of multi-dimensional fluorescence imaging, photodynamic therapy, three-dimensional (3D) data storage and 3D micromachining. Its advantages over single photon absorption include deeper sample penetration, better spatial selectivity and resolution, and reduced photochemical damage/decomposition and reduced light scattering at longer wavelengths.^{1, 2} Although applications based on two-photon excitation have matured considerably, they are primarily based on excitation wavelengths ranging from 700–1100 nm, which sets an upper bound on the sample penetration depth, especially for bioimaging applications.³ For deeper penetration, longer wavelengths over the optical windows in the water attenuation spectrum (~1650–1850 nm and ~2100–2300 nm) are desired for bioimaging. Specifically, fluorophores or fluorescent labels with large multiphoton absorption cross-sections (σ_n) and high photoluminescence quantum yield (PLQY, η) are essential for multiphoton microscopy (MPM). The figure of merit is known as the multiphoton action cross-section ($\eta \times \sigma_n$).

Amongst the suitable media, traditional inorganic semiconductor nanocrystals (NCs) and organic chromophores exhibit superior performances with relatively large $\eta\sigma_2$ & $\eta\sigma_3$.^{1, 4–7} Unfortunately, traditional semiconductor NCs face considerable challenges at higher order four-photon (4P) and five-photon (5P) excitations due to their relatively small $\eta\sigma_n$ ($n \geq 4$); thereby requiring large excitation fluences that could easily induce sample damage. To date, reports on 4P excited upconversion emission from traditional NCs are few and far between. Specially designed organic dye molecules could achieve up to five-photon excited luminescence,⁸ but their relatively low multiphoton action cross-sections and the quenching effect in water limit practical applications. Hence, fluorophores with even higher-order multiphoton excitation activity are highly desired to transcend this limitation.

Halide perovskite NCs recently emerged as a distinct class of semiconductor NCs could fill this gap. They possess the general ABX_3 formula, where the A-site is a monovalent cation (e.g., $CH_3NH_3^+$ (MA), $HC(NH_2)_2^+$ (FA), Cs^+ , etc.), B-site is a divalent metal cation (e.g., Pb^{2+} , Sn^{2+} , etc.) and X-site is a halide anion (e.g., I^- , Br^- , Cl^- , etc.), these materials have found widespread applications in the fields of photovoltaics, photodetectors, and light emitting devices etc.^{9, 10} Of late, they have demonstrated great potential for MPE applications with their giant nonlinear optical action cross-sections. For instance, the five-photon action cross-section of the core-shell type perovskite NCs is at least 9 orders larger than those of specially designed dye molecules.¹¹ Herein, this article examines the latest developments in halide perovskite NCs for multiphoton applications and presents our perspective on the outlook of these frontier materials for MPM.

Motivation for multiphoton excited bioimaging

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For *in vivo* photoluminescence (PL) imaging, the imaging depth and clarity face two main constraints: one is having sufficient light excitation to reach the focal plane through the turbid biological tissue and grapple with the signal-to-background ratio (SBR) of the excitation in scattering biological tissue; the second is requiring the photons emitted by the fluorescent labels to penetrate the tissue to the collection optics and content with the effect of tissue autofluorescence. Firstly, the choice of the excitation spectral window is determined by the light scattering and absorption of the imaging tissue. Taking mouse brain tissue as an example, Fig. 1a shows the wavelength-dependent water absorption mean-free-path length (l_a),¹² calculated tissue scattering mean-free-path length (l_s) and their combined effective attenuation length (l_e). The wavelength-dependent scattering length is provided by the empirical formula¹³ $l_s = (1000/4.72) \cdot \lambda^{2.07}$; and the combined effective attenuation length⁵ $l_e = (l_a^{-1} + l_s^{-1})^{-1}$. It is evident that longer excitation wavelengths generally provide greater penetration. Considering the effective attenuation average free path length, the optimal excitation wavelength window is around 1700 nm.

However, even from the excitation viewpoint, the imaging depth and clarity are not only simply related to the penetration ability of the excitation light, but more importantly, it also depends on the SBR of the excitation, which is closely related to the order of multiphoton excitation. The signal generated in the scattering media for MPM is approximately:^{5, 14}

$$S_n = \frac{a_n \pi^{n-3} N A^{2n-4}}{8 \lambda^{2n-3}} C_n n_0 \langle P \rangle^n e^{-\frac{nz}{l_e}} \quad (1)$$

And the background from the sample surface within one effective attenuation length is given by:⁵

$$B_n \approx \left(\frac{n_0^2}{\pi z^2 N A^2} \right)^{n-1} C_n \langle P \rangle^n l_e \quad (2)$$

The signal-to-background ratio is therefore derived as:

$$SBR_n = S_n / B_n \approx \frac{\pi^{2n-4} a_n N A^{4n-6} z^{2n-2}}{8 n_0^{2n-3} \lambda^{2n-3} l_e} e^{-\frac{nz}{l_e}} \quad (3)$$

where $a_n = 70, 28.1, 18.3$ and 13.2 corresponding to n (order of MPE) = 2, 3, 4 and 5, respectively;¹⁴ NA is the numerical aperture of the focusing lens; λ is the excitation wavelength; C_n is the constant when considering the same order (n) of multiphoton excitation process, and the same experimental conditions and it includes the contributions of PL label's concentration, PLQY, σ_n , etc.; n_0 is the index of refraction of the imaging medium; $\langle P \rangle$ is the time-averaged power of the excitation beam; and z is the imaging depth. Fig. 1b shows a comparison between the SBR for 2PM (800 nm), 2PM (1200 nm), 3PM (1200 nm), 3PM (1700 nm), 4PM (1700 nm) and 5PM (2200 nm) excitations, with $NA = 1$ and $n_0 = 1.33$ for water-based medium. It is clear from Fig. 1b that longer excitation wavelength itself offers scant improvements in MPM SBR at the same multiphoton order. However, higher-order multiphoton excitation provides a significant improvement in SBR over their lower-order counterparts even when at the same excitation wavelength. According to equation 2, the background signal of MPE drops by $\sim 1/z^{2n-2}$. Therefore, compared to lower-order

MPE, high-order MPE greatly reduces the out-of-focus background of the area away from the focal plane, thereby improving SBR by several orders of magnitude. For example, at a depth of 2 mm, 5PM excitation (2200 nm) yields 8 orders higher SBR than 3PM excitation (1200 nm). It is worth noting that for the 1700 nm excitation light with large l_e value (about 462 μm obtained from Fig. 1a), the imaging depth of 3PM and 4PM in the range of 700-4000 μm has considerable SBR. Especially for 4PM, its SBR at 4 mm is at least three orders of magnitude larger than 5PM (2200 nm).

In addition to the excitation window and the order of multiphoton excitation, the emission window of the fluorescent label is also very important for deep imaging (few millimetres) of biological tissue. Given that the emitted photons may also be absorbed and scattered by the sample, the detection depth will also be limited if the emitted photons cannot reach the detector through the sample depth. In addition, the autofluorescence from biological tissues also poses a challenge. Fig. 1c, d shows the wavelength-dependent attenuation coefficient curve of the mouse brain tissue model (including tissue model,¹³ main endogenous biomolecules: oxygenated and deoxygenated haemoglobins,¹⁵ and water^{12, 16}) in the visible to near infrared (NIR) range, and representative normalized autofluorescence spectra of tissues (brain¹⁷ and skin¹⁸). Fig. 1c shows that compared to visible light (green), the NIR attenuation by biological tissue is reduced by approximately 7 times. In addition, as shown in Fig. 1d, the autofluorescence of the representative skin and brain tissue mainly occurs in visible light (425-650 nm) and is negligible at longer wavelengths.

Given that the mouse skull cavity is typically around 7-8 mm, existing 2PM can only image less than 10% depth of the mouse brain (after skull removal) due to strong absorption and light scattering by the tissue. A breakthrough⁵ in imaging depth (~ 1.4 mm) was achieved using 3-photon excitation (at 1700nm) with careful sample preparation and D₂O to suppress the 1675 nm H₂O absorption bands. Nevertheless, the imaging depth is <2 mm required for below scalp through skull imaging. Higher order multiphoton excitation (>4PM) offers an attractive opportunity for truly non-invasive imaging of tissue morphology and physiology for mice cerebral dysfunction studies. Previously, this was severely hampered by the lack of suitable fluorescent labels. But this limitation could possibly be transcended with perovskite nanocrystals. Currently, much research is focused on the multiphoton excitation of bromide perovskite nanocrystals, whose emission is green (~ 520 -550 nm). Therefore, it is desirable to adjust the emission wavelength of multiphoton excited nanocrystals for biological imaging to NIR wavelengths.

The core/shell or medium/matrix process for MPE perovskite NCs

Compared to bulk materials, NCs are expected to have higher multiphoton absorption (MPA) capacities due to their strong dielectric confinement and surface enhancement effects.¹⁹⁻²¹

On this basis, the core/shell or medium/matrix hybrid structure can provide/promote effective passivation of non-radiative surface traps, local field effects, antenna-like effects, internal field photo-induced screening and protection etc.,^{11, 22–26} which can further enhance the nonlinear optical absorption performance and stability of confined NCs. Perovskite NCs with core/shell structure are usually synthesized using modified ligand-assisted reprecipitation (LARP) techniques. The LARP growth method was first proposed by Zhang and coworkers in 2015 for convenient and universal synthesis of MAPbX₃ colloidal quantum dots (QDs).²⁷ The basic principle of the LARP process is supersaturated recrystallization in the presence of ligands, in which the supersaturated state can be achieved by changing the temperature, evaporating the solvent, or adding a miscible solvent with low ion solubility. Fig. 2 illustrates the possible core/shell or medium/matrix process for the preparation of MPE perovskite NCs.

When the LARP technique is applied in core/shell perovskite NCs, the shell is prepared by a one-pot or post-synthesis method.^{11, 28, 29} In a typical one-pot synthesis, the required precursor salt is generally dissolved in a good polar solvent such as dimethylformamide (DMF), dimethylsulfoxide (DMSO), acetonitrile (ACN) etc., is then dropped into a poor/non-polar solvent (such as toluene or hexane) in the presence of ligands and the shell precursor. Ligands used are often organic ammonium ions and/or carboxylic acids and are therefore usually dissolved in polar solvents as mentioned above. Precursors corresponding to different shell materials are selectively present in polar or non-polar solvents according to their chemical characteristics. For example, a layered structure of perovskite (OA)₂PbBr₄ (OA = C₈H₁₇NH₃⁺) is used as a shell with its precursor salt dissolved in the polar solvent of MAPbBr₃ precursor can eventually synthesize the type I core-shell multi-dimensional perovskite MAPbBr₃/(OA)₂PbBr₄ NCs with size of 9–10 nm (Fig. 3a,b).²⁸ but when an oxide such as silica is used as a shell, its precursor tetramethoxysilane (TMOS) is dissolved in the non-polar solvent.²⁹ Transmission electron microscope (TEM) and high-angle annular dark-field scanning transmission electron microscope (HAADF-STEM) images clearly show the successful preparation of monodisperse core-shell NCs CsPbBr₃@SiO₂ (Fig. 3c,d). Interestingly, Huang *et al.* recently also realized the monodisperse core/(multi)shell nanostructure of MAPbBr₃@SiO₂/PVDF by immersing the pre-prepared hollow silica nanospheres in a mixed solution of PVDF and MAPbBr₃ precursor.³⁰ To achieve the core-shell structure in post-synthesis, the typical method is to place the perovskite NCs pre-synthesized by LARP in a new shell reaction system, for example, a toluene solution in which titanium butoxide is dissolved is added dropwise to the perovskite NCs colloids, followed by hydrolysis, drying, and calcination to obtain core/shell nanostructures of perovskite/TiO₂.³¹ For the post-synthesis approach, it is difficult to avoid damage to the pre-synthesized perovskite NCs during the shelling process. As perovskite materials are usually very sensitive to polar solvents, it is impossible to perform the shelling process in polar solvents, which further limits the type of shell materials.

When multiple NCs are encapsulated in a shell, the shell acts as a matrix, in which NCs are dispersed as the media. Such a medium/matrix system is not only obtained via the modified synthesis of the above-mentioned core/shell system,^{32–35} but is also realized by host-guest hybridization of porous materials. For example, a commercially available synthetic porous inorganic material such as mesoporous silica/alumina is used as a template,^{36–39} and a perovskite precursor solution is inserted into the template pore/channel to form a perovskite NCs dispersed medium/matrix hybrid structure. Commercial mesoporous inorganic templates provide the physiochemical ruggedness and pore/channel structures of different sizes, which facilitates the preparation of NCs of different sizes in the template structure. Recently, He *et al.* used a metal-organic framework (MOF) microporous single crystalline material ZJU-28 and adopted an *in-situ* growth strategy to preliminarily use the framework micropores to adsorb Pb²⁺ ions and then immerse the Pb²⁺ contained MOF single crystal into a MABr solution. By controlling the diffusion and combination of Pb²⁺ ions and MABr in the framework, confined *in-situ* nucleation growth of perovskite MAPbBr₃ QDs in a MOF single crystal is achieved, with crystal color changing from colorless to yellow under the bright field, and emission color changing from dark-blue to the characteristic green of MAPbBr₃ (Fig. 4).²⁵ It is worth noting that because the coordination bond strength between organic ligands and metal junctions in MOF is generally not high enough, MOFs with too rigid structures are vulnerable to damage when growing QDs/NCs *in-situ*.

Giant multiphoton action cross-sections in perovskite NCs

The multiphoton action cross-section is the product of the photoluminescence quantum yield (PLQY, denoted η) with the MPA cross-section (σ_n , n is the number of photons absorbed simultaneously), and is a direct characterization of the multiphoton excited luminescence brightness.⁴⁰ The absolute PLQY of perovskite QDs is relatively easy to measure using a commercial/home-built system combining a spectrometer with a BaSO₄-coated integrating sphere excited by a light source at certain wavelength. MPA cross-sections can usually be obtained using the open-aperture Z-scan,^{41, 42} static intensity-dependent transmission (SIDT) measurement^{43, 44} and/or multiphoton excited photoluminescence (MEPL) ratio method.^{40, 45} The typical steps are as follows:

(i) To determine the single-photon absorption cross-section σ_1 . The Poisson distribution, $P_k = e^{-\langle N \rangle} \langle N \rangle^k / k!$, describes the absorption probability of a certain number of photons (k) at an average number of photons absorbed ($\langle N \rangle$), where $\langle N \rangle$ is defined to be the product of σ_1 (in cm²) and the photon fluence (J , in photons/cm²), that is, $\langle N \rangle = \sigma_1 J$. By analysing the bleaching signal at the exciton absorption peak in the transient absorption (TA) data of the optically diluted sample, the

following equation can be obtained using the Poisson distribution:⁴⁶

$$|\Delta OD(t_l)|_{norm} = 1 - P_0 = 1 - e^{-\sigma_1 J} \quad (4)$$

in which, $|\Delta OD(t_l)|_{norm}$ refers to the normalized TA signal at the exciton absorption peak with pump photon fluence dependence at a sufficiently long pump-probe delay time (t_l), and P_0 refers to the unexcited probability for the pump pulse with a certain photon fluence J . A sufficiently long t_l is to ensure the completion of the Auger decay and the dynamic process of multiexciton participation, leaving only TA signals reflecting the single exciton or single photon participation. By plotting J and $|\Delta OD(t_l)|_{norm}$ and fitting the data using Equation 4, the σ_1 value can be derived. This method does not require information such as the size and concentration of NCs and is a relatively accurate means of obtaining single photon absorption cross-sections. In addition, the value of σ_1 can also be estimated by methods such as single-dot spectroscopy, local field effects, etc.^{47, 48}

(ii) To determine the concentration of NCs by the Beer-Lambert law:

$$A = \alpha l \rho = \left(\frac{N_A}{\ln 10} \right) \sigma_1 l \rho \quad (5)$$

Here, A is the absorbance, α is the molar absorption coefficient (in $\text{L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$), l is the optical path length (in cm), ρ is the concentration of NCs (in $\text{mol} \cdot \text{L}^{-1}$) and N_A is Avogadro constant. In the case of known σ_1 , the concentration of the sample can be deduced by measuring the absorbance of the sample at the pump wavelength position by using ultraviolet/visible (UV/Vis) absorption spectrum. However, for systems with high concentration and strong optical absorption, Equation 5 is less reliable. Methods such as elemental analysis and energy dispersion spectroscopy are required for estimation.⁴⁶

(iii) To determine σ_n ($n \geq 2$) by using open-aperture Z-scan measurement, SIDT or MEPL ratio method. Generally, the two-photon absorption (2PA) coefficient β and three-photon absorption (3PA) coefficient γ can be obtained easily by Z-scan or SIDT measurements. But it should be noted that the Z-scan measurement is only suitable for samples with weak scattering, otherwise the measurement result will be overestimated due to significant contributions from scattering. In order to make a more accurate evaluation of strong scattering samples, a static intensity-dependent transmission measurement was developed on the basis of Z-scan, that is, the sample is kept at the waist of the laser beam, and nonlinear transmission and correlated coefficients were obtained by simultaneously monitoring a series of incident intensity and transmitted intensity. The fixed spot size avoids the introduction of nonlinear scattering, so that the nonlinear transmission can be more accurately attributed to the contribution of nonlinear absorption.²¹ Then σ_2 and σ_3 can be obtained according to $\beta = \rho \sigma_2 / (\hbar \omega)$ and $\gamma = \rho \sigma_3 / (\hbar \omega)^2$ where $\hbar \omega$ is the incident photon energy. Due to the limited detection efficiency of the open-aperture Z-scan/SIDT measurement and the high excitation peak intensity required (especially for higher-order

MPA processes), it is easy to cause negative effects such as sample damage and artefacts, so this method is rarely used for higher-order MPA characterization.¹¹ As an alternative, the MEPL ratio method can provide much higher detection sensitivity, which is particularly beneficial for the characterization of high-order MPA properties. The multiphoton (including single photon) excited photoluminescence (PL) strength F_n can be derived as:

$$F_n = C_{n,\emptyset,L,\tau,I,w} \rho \eta \sigma_n \quad (6)$$

where $C_{n,\emptyset,L,\tau,w}$ is the coefficient dependent on n (a positive integer), the MEPL collection efficiency of the experimental setup ($\emptyset$), the sample thickness (L), the laser pulse width (τ), the laser intensity at the sample position (I) and the beam waist at the sample point (w). There are some reports on the derivation of the coefficient $C_{n,\emptyset,L,\tau,w}$ and its exact expression in specific systems.^{11, 25, 45, 49} Through step (i) or open-aperture Z-scan/SIDT measurement to obtain the σ_1 or σ_2 of the sample, and then using Equation 6 to compare the PL integrated intensity of different multi-photon excitation processes of the same sample, σ_n can be obtained. In addition, the σ_n of the sample can also be obtained by comparing the multiphoton excited PL integral intensity of the sample with a reference material of known ρ , η , σ_n .²⁵ Obviously, the reliability of the results obtained by the MEPL ratio method largely depends on the accuracy of the relevant parameters of the reference sample. Overall, both the open-aperture Z-scan measurement, SIDT measurement and the MEPL ratio method require calibration/verification by a standard sample in order to obtain more accurate results.

Most metal halide perovskites have intrinsically large optical absorption properties due to their characteristics of direct gap nature and relatively high joint density of states (JDOS) near the fundamental electronic absorption edge.⁵⁰ Probably due to the relatively stable structure and performance, many recent studies have focused on the Cs-based lead halide perovskite system and confirmed that it generally has very high multiphoton absorption cross sections.⁵¹⁻⁵³ It is worth noting that the reported three-photon absorption cross section of CsPbI₃ QDs can reach $\sigma_3 \sim 1.1 \times 10^{-73} \text{ cm}^6 \text{s}^2 \text{photon}^{-2}$ (~ 1400 - 1500 nm excitation),⁵³ which is about 4 orders of magnitude higher than conventional CdSe red-emission QDs ($\sigma_3 \sim 2.8 \times 10^{-77} \text{ cm}^6 \text{s}^2 \text{photon}^{-2}$), and is ~ 7 orders of magnitude higher than the best organic molecule IPPS ($\sigma_3 \sim 3.7 \times 10^{-80} \text{ cm}^6 \text{s}^2 \text{photon}^{-2}$).⁸ Excitingly, Chen *et al.*¹¹ discovered that core/shell perovskite NCs (MAPbBr₃/(OA)₂PbBr₄ dispersed in toluene) can exhibit up to five-photon excitation process (i.e., absorb up to five low-energy photons simultaneously and then emit one high-energy photon, see Fig. 5a,b) with very large multiphoton absorption characteristics, and their advantages become more distinct as the number of multiphoton orders increases. For example, its $\eta \sigma_2$ and $\eta \sigma_3$ values are 2-3 orders of magnitude higher than the best performing conventional inorganic semiconductor NCs and organic molecules, and its $\eta \sigma_4$ value is approximately 3-5 orders of magnitude higher than the result of best reported organic chromophore, while amazingly its $\eta \sigma_5$ value

($\sim 10^{-136} \text{ cm}^{10} \text{ s}^4 \text{ photon}^{-4}$, Fig. 5c) is more than 9 orders of magnitude larger than state-of-the-art specially designed organic molecules⁸. In addition, He *et al.* also achieved comparable results in the medium/matrix typed MAPbBr₃-QDs/MOF solid-state single crystal system.²⁵ The giant multiphoton absorption cross-section of the core/shell or medium/matrix systems arises due to several possible reasons. Firstly, the increased PL lifetime (Fig. 5d,e) in MAPbBr₃/(OA)₂PbBr₄ (or MAPbBr₃-QDs/MOF system) compared to the core-only MAPbBr₃ indicates that the shell/matrix can provide effective surface passivation for the core/medium, reducing non-radiative surface traps, thereby increasing the probability of multiphoton transitions.^{22, 23} Secondly, the unique combination of core/shell or medium/matrix with a large difference in dielectric constant gives rise to local field effects and thus helps to enhance nonlinear optical performance.^{23, 54} Thirdly, the good spectral overlap (Fig. 5f) between the core absorption and the shell emission as well as the close contact between the core and shell are favourable for non-radiative energy transfer (antenna effect) from the shell to the core, which is beneficial for enhancing the optical absorption.^{24, 55} Besides, light-induced screening (during photoexcitation) of the internal electric field caused by lattice mismatch between the core and shell may also be a source of σ_n enhancement.⁵⁶ However, theoretical research on the extremely high MPA characteristics of metal halide perovskite NCs is still lacking, which should be a focus for future works.

For non-linear absorption characterization of perovskite materials, one must also take note of the size effects. Like many other semiconductor materials, the non-linear absorption capacity of perovskite micro-nano structure is generally several times larger than that of the large-scale bulk counterpart. For example, according to reports from Han's research group and their coworkers, the two-photon coefficient of CsPbBr₃ quantum dots⁵⁷ or microcrystals⁵⁸ is $\sim 10\text{--}11 \text{ cm GW}^{-1}$, which is about 3 times larger than that of centimeter-level CsPbBr₃ single crystals (3.7 cm GW^{-1})⁵⁹. In addition, for the nanoscale range that we are interested in perovskite materials that exhibit regular size-dependent nonlinear absorption characteristics. Within a certain range (e.g., 4–12 nm), the two-photon absorption cross section of CsPbBr₃ quantum dots has a linear relationship with the cubic of the particle size (the reported power exponent is 3.3 ± 0.2)⁵⁷. This implies that within the acceptable range of imaging resolution, it is more advantageous to select perovskite nanoparticles with a larger average particle size as the imaging label.

Stability improvements of perovskite NCs

The instability of metal halide perovskites has always been their Achilles heel. Based on current knowledge, its instability arises from intrinsic factors related to structure and chemical composition, as well as external factors that cause decomposition such as light, oxygen, moisture (polar solvent atmosphere), and temperature.^{60, 61} Although the high surface energy contributes to the stability of perovskite nanostructures

to a certain extent, the large specific surface area and reactivity make nano-perovskite materials more easily decomposed by external adverse factors. Moreover, the actual imaging application environment is usually a water-based cell fluid or biological culture solution, which will bring about more daunting challenges to perovskite nanomaterials. Furthermore, halide perovskites contain Pb and heavy metals that are toxic to cells.

There has been much effort devoted to stabilizing the structure and properties of perovskite NCs through some form of encapsulation. There is some success and could possibly help mitigate some of the challenges for bioimaging applications. Huang *et al.* realized encapsulation of MAPbBr₃-QDs in a SiO₂ spherical matrix (PLQY is about 87%) and maintained 94.10% of the initial PL after 7 hours of continuous excitation at 60% relative humidity.³² After that, Zhong *et al.* modified the synthesis method to successfully achieve a core-shell hybridization of CsPbBr₃@SiO₂ at the single particle level and obtained luminescence stability that can even be sonicated in water (Fig. 6a).²⁹ However, the dispersibility of the nanoparticles in water is poor because the surface is covered with oleic acid (OA) and oleylamine (OAm). Subsequent efforts should focus on developing methods to modify the surface of this type of nanoparticles to obtain good dispersibility. The multi-core/single-shell CsPbBr₃ hybrid system seems to show better performance. For example, after CsPbBr₃@SiO₂ nanoparticles prepared by Li *et al.* were immersed in water for 13 hours, their PLQY could still maintain $\sim 80\%$ of the initial value. And after immersing in water for 15 days, two-photon pumped random lasing can still be obtained.³⁵ CsPbBr₃@PMMA nanoparticles prepared by Wang and coworkers can maintain $\sim 81\%$ of the initial emission intensity after 80 days of immersion in water.³³ However, it remains a challenge to reduce the size of these nanoparticles ($> 100 \text{ nm}$) whilst maintaining the good performance. For the MAPbBr₃/(OA)₂PbBr₄ system with high multiphoton action cross-sections, Vijila *et al.* used PMMA to coat the MAPbBr₃/(OA)₂PbBr₄ nanocrystals, achieving a hybrid film with high stability where the film retains a PLQY of $\sim 83\%$ after 18 h immersion in water.⁶² However, this processing approach must still be adapted for individual nanoparticles rather than thin-film morphology. He *et al.* realized multiphoton cross sections (from 1PE to 5PE) comparable to the record values of MAPbBr₃/(OA)₂PbBr₄ solution system in a solid-state medium/matrix system of MAPbBr₃-QDs/MOF at a relative humidity of 60%.²⁵ Under the same humidity conditions, the system also exhibited good multiphoton excited photo-stability, that is, upon continuous excitation of 1-photon (480 nm), 2-photon (960 nm), 3-photon (1440 nm), 4-photon (1800 nm) and 5-photon (2100 nm), it still maintained 94% (100 min for 1PE), 94% (100 min for 2PE), 86% (100 min for 3PE), 75% (60 min for 4PE), and 55% (60 min for 5PE) of the initial PL, respectively (Fig. 6b,c). Compared to a dense oxide shell, although the open MOF network structure with adjustable physiochemical properties has great potential although or highly selective/sensitive sensing, it is difficult to simultaneously provide sufficient protection against high humidity. One possible solution is to

develop or select MOF materials with hydrophobic properties, and the MOF size is preferably reduced to the nanoscale.

Recently, You et al. used the atomic layer deposition (ALD) method to deposit AlO_x shells on the basis of in-situ crystallization of Cs-based perovskite nanocrystals in mesoporous silica nanospheres to realize hydrophilic water-resistant perovskite-based nanomaterials.⁶³ Despite the lack of information about its PLQY, the material's good dispersibility in water as well as its obvious luminescence in boiling water and many polar solvents are still worthy of follow-up studies. The synthesis of existing perovskite nanocrystals usually uses long hydrocarbon (or lipophilic) chain amines and acids as ligands. On one hand, the exposure of the lipophilic end is not conducive to the dispersion of nanoparticles in water; while on the other hand, these ligands are prone to proton exchange and are easily separated from the surface, thereby reducing the luminescent performance of the material.^{64, 65} In the latest report, Liu et al. developed a surface carboxylate passivation method on CsPbBr_3 NCs in the absence of amines and acids, thereby inhibiting proton exchange.⁶⁶ The resultant CsPbBr_3 surface passivation product (PLQY ~64%–71%) can still maintain about 90% of the initial intensity when stored in ethanol for 30 days. In addition, the sample has an estimated lifetime of more than 2000 h under continuous blue light irradiation of 10 W/cm^2 in the air, which is comparable to the photo-stability of commercial CdSe quantum dots. In addition to the construction of an additional protective shell or matrix layer to improve the water resistance of perovskite NCs, not long ago, Liu and his coworkers reported an important phenomenon, that is, the perovskite NCs synthesized in an alkaline atmosphere can form a perovskite@ PbBr(OH) structure in water, thereby greatly improving PLQY (from 2.5% to 71.54%) and photo-stability.⁶⁷ The significant improvement in luminescence and stability stems from the fact that PbBr(OH) shell passivates the defects of the perovskite NCs, confines carriers within the NCs, and prevents the NCs from being exposed to air and moisture. The resulting perovskite@ PbBr(OH) structure still possesses 64.28% PLQY after one year in water, and this strategy is universal to MAPbBr_3 , FAPbBr_3 and CsPbBr_3 . These studies will greatly enhance the application potential of perovskite-based NCs in water-based imaging systems.

Future outlook

Halide perovskite NCs represent one of the most exciting new developments for multiphoton applications. In the past few years, many attractive properties have been uncovered, such as extremely high PLQY, broadband tuneable emission wavelengths, and giant multiphoton absorption/action cross sections. Table 1 summarizes the most promising perovskite-based nanoparticles and compares them with some well-researched fluorescence labelling materials such as dye molecule PREX 710⁶⁸, upconverting nanoparticle $\text{NaYF}_4\cdot\text{Yb}$, Er⁶⁹, and core-shell QDs CdSe/ZnS ^{70, 71}. It is exciting that these reported perovskite-based NCs generally show the advantage of high PLQY in the water environment. In addition, lead halide

perovskite NCs have been proven to possess high-order ($n>3$) multiphoton absorption emission capability, which is also lacking in many existing fluorescence labelling materials. These two properties present a distinct advantage for perovskite-based NCs in the field of bioimaging. However, there are still many pertinent issues in halide perovskite fluorophores that must be addressed for any *in vitro* and *in vivo* testing. These include: greatly improving the stability (and simultaneously addressing the toxicity) of perovskite NCs in water-based solutions through encapsulation while maintaining high MPA and emission performance, the development of stable perovskite NCs with red or near-infrared emission, the hybridization and multi-functionalization of perovskite NCs based materials, and the theoretical study of the extremely high MPA properties of perovskite NCs.

The development of lead-free perovskite nanomaterials is also a very important research topic, because it can resolve the toxicity problem of lead perovskite materials. So far, Bi-based, Sb-based, Cu-based and double perovskite NCs have been proven to possess excellent air stability.^{72, 73} For example, $\text{Cs}_3\text{Bi}_2\text{Br}_9$ NCs,⁷⁴ $\text{Cs}_3\text{Sb}_2\text{Br}_9$ QDs,⁷⁵ $\text{Cs}_2\text{AgBiBr}_6$ double perovskite NCs⁷⁶ and Cs_2CuBr_4 QDs⁷⁷ can be stable in air for at least a month. Although the stability of the pure Sn-based perovskite system is poor, its water resistance can still be improved by additional metal (Bi^{3+} , Sb^{3+} and so on) doping.^{78, 79} Recently, Tan et al. successfully prepared $\text{Cs}_2\text{Sn}_{1-x}\text{Te}_x\text{Cl}_6$ lead-free perovskite crystals with PLQY up to ~95.4%, which were immersed in deionized water for 6 hours without loss of PL intensity, and still maintained strong yellow-green emission light.⁸⁰ However, if it is to be used in the field of bioimaging, the preparation of nano-morphology of the material is essential. In addition, the most important challenge for lead-free perovskite systems is that there is still no report on multiphoton absorption/excitation. Therefore, further research in this area is particularly urgent.

Last but not the least, to fully leverage the profound advantages of the “giant” higher order (>3 photon excitation) action cross-sections of halide perovskite NCs for deep (3–4 mm) mouse brain imaging, a radical redesign of the optical systems in existing microscopes is also needed to handle the IR excitation wavelengths (i.e., 1600–2300 nm). This will require engineering of new wide field of view, long working distance objectives with adaptive optics correction. Beyond these material science, physics and optical engineering challenges, the biochemistry and bio-medical aspects in the functionalization of these encapsulated perovskite NCs for labelling of cellular components and extensive cytotoxicity and photo-toxicity tests in cells and organisms etc. are also urgently needed – a truly multi-disciplinary effort indeed! Nonetheless, with the advances in chemical synthesis and manipulation of these perovskite nanomaterials, the stability and toxicity issues for bio-imaging are certainly surmountable and is an emerging area to watch out for exciting new developments in multi-photon applications.

Conflicts of interest

There are no conflicts to declare.

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Figure Captions

Fig. 1 (a) Attenuation spectrum of a (mouse) brain tissue model based on Mie scattering and water absorption, showing the absorption length of water¹² (l_a , olive dash line), the scattering length of brain cortex¹³ (l_s , yellow dash-dot line), and the combined effective attenuation length (l_e , red solid line).⁵ (b) Calculated signal-to-background ratios (SBRs) of 2PM at 800 nm, 2PM and 3PM at 1200 nm, 3PM and 4PM at 1700 nm and 5PM at 2200 nm at NA = 1, respectively. (c) Attenuation spectrum of a mouse brain tissue model in the visible to NIR range based on Mie scattering, water absorption and the absorption of endogenous biological molecules, showing the absorption coefficient of water^{12, 16} (olive short-dash-dot line), the scattering coefficient of brain tissue¹³ (orange dash-dot line), the absorption coefficient of the two main endogenous biological molecules: oxygenated- (yellow short-dash line) and deoxygenated haemoglobins (light olive short-dash line), and the combined effective attenuation coefficient (red solid line). Inset: the enlarged view of the water absorption spectrum. The blue stars reveal approximately 7 times attenuation reduction in the NIR region compared to visible green light. (d) Autofluorescence of the representative tissues skin¹⁸ (red short-dot line) and brain¹⁷ (olive short-dash line), showing the autofluorescence mainly occur in the visible region (425–650 nm) and become negligible at longer wavelengths.

Fig. 2 A schematic illustration of core/shell or medium/matrix process for MPE perovskite NCs.

Fig. 3 (a) Schematic diagram of the core-shell multidimensional perovskite NCs with 3D MAPbBr₃ as core and 2D (OA)₂PbBr₄ as shell, and their type-I energy level alignment. (b) 1PA spectra and one-photon excited PL spectra of the MAPbBr₃/(OA)₂PbBr₄ NCs with their representative TEM images (scale bar, 10 nm) shown in the inset. (c) TEM image of synthesized CsPbBr₃@SiO₂ core-shell NCs. (d) HAADF-STEM image and elemental mapping images showing the elemental distribution of Si, Cs, Pb, Br and the overlapped image. Scale bars, 10 nm. Panel (a) and (b): adapted with permission from ref. 11; Copyright © 2017, Springer Nature. Panel (c) and (d): adapted with permission from ref. 26; Copyright © 2018, American Chemical Society.

Fig. 4 (a) The SEM image of a single ZJU-28@MAPbBr₃. Scale bar, 10 μm. (b) The STEM image of ZJU-28@MAPbBr₃. Scale bar, 100 nm. (c) The optical image of a single ZJU-28 under bright field. Scale bar, 10 μm. (d) The optical microscopy image of a single ZJU-28@MAPbBr₃ under bright field, scale bar, 10 μm. (e-f) Optical images of a single ZJU-28 (e) and ZJU-28@MAPbBr₃ (f) upon excitation of 365 nm. Scale bar, 20 μm. Panel (a) to (f): reproduced with permission from ref. 23; Copyright © 2018 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

Fig. 5 (a) Schematic diagram of the 5-photon excitation process in perovskite NCs. (b) Slopes n plotted as a function of laser excitation wavelength (photon energy), where n is the number of photons involved in the MPE process and the MPE PL signal is proportional to the n^{th} power of excitation fluence. (c) Five-photon action cross-section ($\eta\sigma_5$) spectra of the MAPbBr₃, MAPbBr₃/(OA)₂PbBr₄ and CsPbBr₃ NCs. Error bars indicate experimental uncertainty of $\pm 15\%$. (d) Single- and multi-photon excited PL decay curves in MAPbBr₃ NCs. (e) Longer single- and multi-photon excited PL decay lifetimes are obtained for MAPbBr₃/(OA)₂PbBr₄ core-shell NCs than for MAPbBr₃ NCs. (f) Large spectral overlap between the emission of (OA)₂PbBr₄ thin film and the excitation of MAPbBr₃ NCs. Panel (a) to (f): adapted with permission from ref. 11; Copyright © 2017, Springer Nature.

Fig. 6 (a) Single-photon excited photostability of CsPbBr₃ and CsPbBr₃@SiO₂ under ultrasonication upon ultra-violet (UV) light irradiation. (b) Continuing illumination-time-dependent luminescence intensity spectrum of the single ZJU-28-MAPbBr₃ crystal upon 436 nm light exposure. Scale bar, 5 μm . (c) Multiphoton-excited photostability of a single ZJU-28-MAPbBr₃ upon femtosecond laser excitation 1440 nm. Test environment: temperature of 27 $^{\circ}\text{C}$, humidity of 60%. Panel (a): reproduced with permission from ref. 26; Copyright © 2018, American Chemical Society. Panel (b) and (c): reproduced with permission from ref. 23; Copyright © 2018 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

Table 1 Some well-researched fluorescence labelling materials and potential perovskite-based NCs for bio-imaging.

Materials	Type	Size	Performance
Rhodamine-typed dye PREX 710 ⁶⁸	Molecule	~1.5 nm	PLQY ~13%; 80% of the initial intensity was retained when labelled HeLa cells for 10 min
Streptavidin-functionalized NaYF ₄ :Yb, Er ⁶⁹	Nanoparticle	22 ± 1 nm	Upconversion luminescence (980 nm excitation); Stably labelled CHO-K ₁ cells for one week
Bio-conjugated CdSe/ZnS ^{70, 71}	Core/shell nanoparticle	Not mentioned	PLQY ~20%-30%; Stably labelled HeLa cells for over one week
CsPbBr ₃ @PMMA ³³	Multi-cores/shell nanoparticle	~112 nm	PLQY ~73%; Over the 80 days, the intensity loss of only 19% in water; Stable emission position
CsPbBr ₃ @SiO ₂ ²⁹	Core/shell nanoparticle	~26 nm	PLQY ~90%; PL intensity is not reduced under ultrasonication treatment for 40 min
CsPbBr ₃ @SiO ₂ ³⁵	Multi-cores/shell nanoparticle	~170 nm	PLQY ~78% (initial) and ~62.4% (13 h in water); two-photon-pumped random lasing could still operate after 15 days in water
CsPbX ₃ @SiO ₂ (X = Cl, Br, I) ³⁴	Multi-cores/shell nanoparticle	~120 nm	PLQY of CsPbBr ₃ /SiO ₂ , and CsPbI ₃ /SiO ₂ composites were determined to be ~84% and ~45%; 40% of initial intensity left after 4 h in water
PMMA-MAPbBr ₃ /((OA) ₂ PbBr ₄) ⁶²	Core/shell-matrix film	~4 nm without PMMA	PLQY ~83% after fully immersed in water for 18 h
MAPbBr ₃ @SiO ₂ ³²	Multi-cores/shell nanoparticle	~470 nm	PLQY ~89% in toluene; 92.13% of initial intensity left after 7 h in 80% humidity
MAPbBr ₃ @SiO ₂ /PVDF ³⁰	Core/multi-shells nanoparticle	~20.7 nm	PLQY ~85.5%; 60% of initial intensity left after 2 h in water
mSiO ₂ -perovskite@AlO _x ⁶³	Multi-cores/shell nanoparticle	80-130 nm	Hydrophilic; Keep luminescing in boiling water and many polar solvents
Carboxylate passivated CsPbBr ₃ ⁶⁶	Nanocrystal	22.7 ± 2.6 nm	PLQY ~64%-71%; ~90% of the initial intensity after 30 days in ethanol; Estimated lifetime of > 2000 h under continuous blue light irradiation of 10 W/cm ² in the air
perovskite@PbBr(OH) ⁶⁷	Multi-cores/shell micro rod	Average diameter: 1.5 µm; Length: 4 µm	PLQY ~64.28% after one year in water; Universal to MAPbBr ₃ , FAPbBr ₃ and CsPbBr ₃

TOC

Halide perovskite nanocrystals show great promise for bio-imaging applications due to their large multiphoton absorption cross-sections and their improved water resistant. This frontier article distills the state-of-the-art in their synthesis and non-linear optical characterization and highlights their opportunities and challenges for application in multi-photon deep tissue imaging.