Precise lysosome pH indicator based on self-decomposable nanoparticles

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Nano Express

Keywords: MB@SiO₂, BPSi, lysosome pH indicator, autophagy

Posted Date: March 10th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-16559/v1>

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Version of Record: A version of this preprint was published on July 8th, 2020. See the published version at <https://doi.org/10.1186/s11671-020-03367-0>.

Abstract

Acidic pH of lysosomes are closely related to the autophagy, thus well known of the precise lysosomes pH changes will give more information on the autophagy process and status. So far, however, only pH changes in a relative broad range could be indicated, the exactly lysosomes pH detection has never arrived. In our study, we established an endo/lysosome pH indicator based on self-decomposable SiO₂ nanoparticle system with specific synthesis parameters. The central concentrated MB in the central-hollow structural nanoparticles presented sensitive release as a function of pH values from pH 4.0-4.8, which is the exactly the pH range of lysosomes. The linear correlation of the OD values and the pH values has been built up, which has been used for detection of lysosomes pH in 6 different cell lines. Moreover, by this system, we succeeded in precise detecting the pH average changes of lysosomes before and after BPSi NPs endocytosis, clarifying the mechanism of the autophagy termination after BPSi endocytosis. So, the self-decomposable nanoparticles based luminal pH indicator may provide a new methodology and strategy to know better of the lysosome pH, then indicate more details on autophagy process or other important signaling about metabolism.

1. Introduction

Lysosomes serve as the final destination for macromolecules, where these macromolecules are degraded by hydrolytic enzymes activated by low pH^[1]. The acidic pH of lysosomes maintained by the vacuolar-type H⁺-ATPase (v-ATPase)^[2] that pumps protons from the cytoplasm into the lysosomal lumen was to keep the activity of ~60 types of hydrolytic enzymes^[3]. Moreover, recent literature reports revealed that acidic pH of lysosomes are closely related to the autophagy^[4], so that well known of the precise lysosomes pH changes will give more information on the autophagy process and status. Based on our studies and literature review, amine positive charged nanoparticle endocytosis will probably increase the pH change in endo/lysosomes, such as primary and secondary amine PEG decorated nanoparticles or some hydrophilic decoration on the particle surface^[5, 6].

The increase pH induced by the endocytosis of amine nanoparticles will dramatic increase in TFEB nuclear localization^[7], results in not only transcriptional upregulation of the pathway, but also causes lysosomal dysfunction, ultimately resulting in blockage of autophagic flux^[7-9].

Thus, in order to predict the autophagy process and the details of autophagy, the lysosome precise pH and its change measurement are very crucial. Till now, from the endo/lysosomes pH value indicating literature reviews^[10] and the commercial products for detected the endo/lysosome pH values, only pH changes in a relative broad range could be indicated, the exactly lysosomes pH detection has never arrived. Thus, to know insight the details of autophagy, establishment of a precise luminal pH changes detection method is an important approach.

In our study, we established an endo/lysosome pH indicator based on self-decomposable nanoparticle systems with specific synthesis parameters. The central concentrated MB in the central-hollow structural

nanoparticles presented sensitive release as a function of pH values from pH 4.0-4.8, which is the exactly the pH range of lysosomes. The linear correlation of the OD values and the pH values has been built up, which has been used for detection of lysosomes pH in 6 different cell lines. Moreover, by this system, we succeeded in precise detecting the pH average changes of lysosomes before and after BPSi NPs endocytosis, clarifying the mechanism of the autophagy termination after BPSi endocytosis. So, the self-decomposable nanoparticles based luminal pH indicator may provide a new methodology and strategy to know better of the lysosome pH, then indicate more details on autophagy process or other important signaling about metabolism.

2. Experimental Section

2.1. BPSi nanoparticles synthesis

The BPSi nanoparticles were prepared by our previous method^[11], and supplied by our collaborator (Wujun Xu, Department of Applied Physics, University of Eastern Finland).

2.2. 10 series self-decomposable nanoparticle systems establishment

The 10 series self-decomposable nanoparticles were synthesized by the methodologies we reported before^[18-21], with modified parameters. In a typical procedure, certain amount of MB was firstly added to a mixture of ethanol (75 mL) with ammonia-water solution (25%, 3.4 mL), after that certain amount of TEOS was added. The series self-decomposable SiO₂-MB NPs were obtained after stirring for 24 hours, and washed 3 times before their being dried. The MB and TEOS amounts added in the protocols were as described in the table 1.

2.3. Cell culture

The cell lines of HCT116, HCT8, HCT15, HepG-2, B16 and A549 were maintained in RPMI 1640 medium (Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2.0 g/L sodium bicarbonate, 0.1 g/L streptomycin sulfate, 0.06 g/L penicillin G and 5.958 g/L HEPES. The cells were maintained in a standard, cell culture incubator at 37°C in a humidified atmosphere with 5% CO₂. All of the NPs were dispersed in the medium by slightly ultrasonication right before their introduction to the cells.

2.4. Characterizations of 10 series self-decomposable nanoparticle systems

The morphology of all series nanoparticles were characterized by HR-TEM with STEM mode, and Si mapping was studied by EDS element mapping. Nanoparticles size distribution analysis was performed by Image J software by calculating the nanoparticles diameters in randomly selected STEM images. Data was analyzed by SPSS15.0 and the statistical results were presented as mean ± S.D.

2.5. The MB loading efficiency and release kinetics study of 10 series self-decomposable nanoparticle systems

In order to study the MB loading efficiency and release kinetics, the standard curve of MB in series concentrations was firstly established. The absorptions of MB was carried out by UV-Vis spectrum with the absorbance at 660 nm, which is the λ_{max} of the monomer MB. The MB loading efficiency was calculated by the equation below, MB loading efficiency(%) = The amount of encapsulated MB/(Total amount of MB input).

The MB release from 10 series nanoparticles were studied in pure water and pH buffers with different pH values (pH 4.0, pH 6.86 and pH 9.18) and Lysozyme Solution (Thermo Scientific™ # 90082). Moreover, the MB release kinetics after specific duration in different pH buffers were also investigated. The OD values at 660 nm and the MB release percentages as a function of time were then studied.

In more details, the MB release studies were performed by the protocols below, dissolved 10 series nanoparticles in 15 mL standard buffer of pH 4.0, 6.86 and 9.18 with lysosome solution, respectively, and performed the MB release in a Hula mixer at 37 °C. During the following 15 days, 1 mL of each sample was collected, then centrifuged at 12000 rpm for 10 min. The supernatant and the precipitate were measured for their absorption spectra at 200-800 nm.

Moreover, the MB release in precise pH buffers with lysozyme solution in the pH range from 4.1-5.5 was also investigated with the same protocols above. Specific time durations (6 hrs, 12 hrs and 24 hrs) were placed as observation time points. The absorptions at 660 nm were recorded in each sample. The linear relationship of absorption of each pH solution and the sum of the squares of the residuals were counted at each time point respectively.

To detect the MB release profiles in cells, HepG-2 cells were cultured in a 75 cm² culture flask, and fed with NPs when the cells were proliferated to 70% of the culture flask. Every 30 mins later, collected the cells. The cells were repeatedly frozen and thawed to release the MB in the cells completely. The cell lysates were centrifuged at 12000 r/min for 10 min. The supernatant was obtained, and measured its absorbance at 660 nm to calculate the total amount of released MB.

2.6. Cellular colocalization of the 10 series nanoparticles and the release performance in 6 different cell lines

Cell TEM was employed to study the colocalization of the nanoparticle in the endo/lysosomes following standard cell TEM protocols. Cells were seeded in the intensity of 1×10^6 cells/flask and incubated for 24 hours, allowing the cell attachment. 10 series of nanoparticles in medium with the same concentration (100 µg/mL) were incubated with the cells for another 12 and 24 hours, respectively. Cells were then washed by PBS for 3 times to remove the excess nanoparticles, then fixed in 2.5% glutaraldehyde solution for longer than 1 day. Fixed cells were then washed and stained by Osmium tetroxide, 1% in deionized water for 1 hours, followed by washed with PBS for 3 times and DI water for 2 times. Classic cell TEM protocol ^[18, 20] was carried out followed by and sections with thickness of 90 nm were collected for TEM observation. MB release as a function of pH values were studied in 6 cell lines with both NPs

$_{6/100}$ and NPs $_{7.5/80}$. Also, the OD values from the release the MB and the MB release percentages were recorded for data analysis.

2.7. Investigation of intracellular uptake of MB@SiO₂ nanoparticles

Live cell confocal microscopy was used to assess the cellular uptake and intracellular fate of the MB@SiO₂ nanoparticles. HepG-2 cells early endosomes were stained (CellLight Early-endosomes-GFP, BacMam 2.0 ThermoFisher Scientific C10586, with excitation/emission $\sim 488/510$ nm) for 16 hrs. And then cells were incubated with NPs $_{6/100}$ (MB excitation/emission: 640/650-700 nm) at the nanoparticles concentration of 100 $\mu\text{g/mL}$ at specific time intervals (2 hrs, 2.5 hrs, 3 hrs, 5 hrs and 6 hrs). Before images were taken, stained the lysotracker with LysoTracker™ Red DND-99 (ThermoFisher Scientific L7528, excitation/emission: 577/590 nm) for 40 mins. After that, remove the stain solution and wash the cells 2-3 times in PBS. Images were taken using a Nikon A1R Confocal Microscope.

2.8. Transcriptome sequencing to evaluate the gene expression change after BPSi feeding

Total RNA extraction of control group and BPSi treated group was performed using the Trizol reagent (Invitrogen, Carlsbad, CA, #15596026) following the standard operating procedures. The quality of the initial total RNA sample for the sequencing experiment was detected using a NanoDrop ND-2000 spectrophotometer. Total RNA that passed the quality control was used in subsequent sequencing experiments. Comparison of the gene expression was performed by next-generation sequencing. All sequencing programs were performed by BGI-Shenzhen Corporation (Shenzhen, China) using the BGISEQ-500 platform. Raw data obtained by sequencing are performed quality control to determine whether the sequencing data is suitable for subsequent analysis. If passed, perform quantitative analysis of genes based on gene expression levels, and perform significant enrichment analysis of GO (Gene ontology) functions on the differentially expressed genes between the selected samples.

2.9. RT-qPCR assay to confirm the activation of TFEB-CLEAR gene network

Total RNA was extracted from the cultured HepG-2 cells of the control and BPSi treated groups by using the Trizol reagent (Invitrogen, Carlsbad, CA, #15596026), and reverse-transcribed to cDNA by using the BioRT Master HiSensi cDNA First Strand Synthesis kit (Hangzhou Bioer technology Co., Ltd) with random primers. cDNA was used to amplify TFEB-CLEAR gene network by quantitative PCR with the Applied Biosystems™ 7500 real-time PCR system (Applied Biosystems, Life Technologies, Carlsbad, CA), with actin as a reference control. Primers used for quantitative RT-PCR were listed in Table S3.

2.10. Western blot assay to confirm that autophagy is activated after BPSi feeding

Cellular proteins of the control group and different concentration BPSi treated groups were extracted by RIPA lysate (Heart biological technology Co., Ltd). The protease inhibitor was added to the RIPA lysate and pre-cooled on ice. Washed the cells 3 times by pre-cooled PBS. Dumped the liquid completely and placed the dish in ice for 2 min. 400 μL of RIPA lysate was added to the surface of the whole dish,

pipetted several times with a pipette, and incubated on ice for 30 min, during which the dish was shaken several times to completely lyse the cells. The lysed cell fluid was transferred to 1ml Eppendorf tubes and centrifuged at 13,000 rpm for 10 min, 4°C. The obtained supernatant was boiled in water for 10 min, and placed in -20°C for later use. Protein concentration was quantified using the Pierce® BCA Protein Assay Kit (Thermo scientific).

Cell extracts containing 25 µg total protein were directly subjected to SDS-PAGE and transferred. The membranes were blocked with 5% skimmed milk and probed with primary antibodies that recognize P62 (Proteintech #18420-1-AP), TFEB (Proteintech # 13372-1-AP), LC 3B (abcam # ab192890), and β-actin (Proteintech #20536-1-AP). Secondary antibodies were chosen according to the species of origin of the primary antibodies and detected by enhanced chemiluminescence (Pierce) or by using the Bio-Rad ChemiDoc XRS+ Gel Imaging System (Bio-Rad, USA). The normalized band intensity of P62, TFEB and LC 3B relative to β-actin was quantified by densitometry using Image J software in the BPSi groups, the data are the mean ± S.D. from three independent experiments.

2.11. Measuring cell pH by PDMPO and pHrodo™ Red Transferrin Conjugate by confocal microscopy

PDMPO study

1×10⁵ HepG-2 cells were cultured on sterile confocal plates overnight and the BPSi nanoparticles were fed with concentration of 100 µg/mL. The next day, before immunofluorescence staining, slides were washed three times with 0.01 M phosphate-buffered saline (PBS), pH 7.4, then added 1µM PDMPO dye (Ex/Em=329/440). After washing with PBS for three times, cells were incubated with fresh RPMI-1640 culture medium, and observed under a fluorescence microscope (Nikon A1R, Japan) with a CCD camera and take pictures within 5 minutes, the ratio of blue and green fluorescence intensity in the lysosomes was then calculated according to the procedure of Chen et al.^[22].

pHrodo™ Red Transferrin conjugate study

HepG-2 cells were plated in confocal plates in the same way for cells attachment for 24 hrs. Then kept plates on ice for 10 minutes. Washed cells with cold Live Cell Imaging Solution (LCIS) containing 20 mM Glucose and 1% BSA. Added pHrodo™ Red Transferrin conjugate (Ex/Em=560/585nm) at 25 µg/mL in LCIS and incubate at 37°C for 20 minutes, then washed cells in Live Cell Imaging Solution (Cat. no. A14291DJ). The observation was also carried out by confocal microscopy. The quantitative analysis of the intensity of the microscopy images was performed by Image J software.

2.12. Detection of cell lysosomal pH

The cells A549, HepG-2, HCT8, HCT15, HCT116 and B16 were cultured in a 75 cm² culture flask, and fed with NPs when the cells were proliferated to 70% of the culture flask. 6 hours later, collected the cells. The cells were repeatedly frozen and thawed to release the MB in the cells completely. The cell lysates were centrifuged at 12000 r/min for 10 min. The supernatant was obtained, and measured its absorbance at

660 nm to calculate the total amount of released MB. The absorbance of the NPs_{6.0/100} at a standard pH was compared to obtain the pH value of each cell.

2.13. Statistical analysis

Statistical analysis was done with the SPSS15.0 software by using analysis of variance (ANOVA) for independent groups. Statistical significance was based on a value of $P < 0.05$.

3. Results And Discussion

We firstly detected differential gene expression when cells fed with the dual PEG functional black porous silica nanoparticles (BPSi NPs) (offered by our collaborating lab^[11]). Based on the Cluster heat map of differential gene expression (Figure S1), we selected more than 2 fold differential gene expressions for further investigation. Both Go (Figure 1a) and KEGG Pathway (Figure S3) classifications were introduced to analyze the differential genes. From the Go (Figure 1b) and KEGG Pathway (Figure S2) enrichment bubble maps, the metabolic and lysosome associated genes including phagolysosome assembly, phagocytosis and xenobiotic metabolic process, etc. were selected for further analysis. Notably, TFEB-CLEAR^[12] associated genes expression were significantly activated. The RT-PCR results (Figure 1c) also verified the gene sequencing results, the genes on TFEB-CLEAR pathway significantly increased, such as CTSD, CTSF, TFEB, MFN1, LAMP2 AND TPP1, etc. ($P_{\text{BPSi VS Control}} < 0.05$). Due to the fact that the major function of TFEB gene is to induce the bio-synthesis of lysosome and promote the occurrence of autophagy^[13]. Western blot analysis was also employed to testify whether the autophagy happened when cells fed with BPSi NPs. From the Western blot results shown in Figure 1d and 1e, TFEB ($P_{80\mu\text{g/ml VS Control}} = 0.000008$), LC3B II/I ($P_{80\mu\text{g/ml VS Control}} = 0.000297$) and p62 ($P_{80\mu\text{g/ml VS Control}} = 0.000016$) proteins all significantly upregulated. As the upregulation of TFEB and LC3B II/I proteins indicating the activation of autophagy^[14], we suspected that the BPSi endocytosis promote the occurrence of autophagy. However, the p62 protein is supposed to be downregulated during the autophagy process, due to the carrier protein nature which brought the endosomes to lysosomes and finally degraded. In our study, the significantly upregulation of p62 indicated the termination of degradation during the endo-lysosome fusion process^[15], which probably caused by pH increase in endo-lysosome vesicles. Thus, the BPSi endocytosis may firstly induced the occurrence of autophagy, then inhibited the autophagy process by increasing the endo/lysosome pH values, owing to its amide alkalinity.

In order to testify the pH increase characteristics in endo/lysosomes by BPSi endocytosis, two commercial pH fluorescent probes were employed in our study, pHrodo™ Red Transferrin Conjugate (Thermo Fisher #P35376) and RatioWorks™ PDMP0.

pHrodo™ Red as a commercial intracellular pH indicator, usually presents weakly fluorescent at neutral pH but increasingly fluorescent as the pH drops. It was supposed to quantify cellular cytosolic pH in the range of 9-4 with a pKa of ~6.5 with excitation/emission of 560/585 nm. We could obtain a qualitative analysis conclusion from 6 cell line determinations that the endocytosis of BPSi NPs has the capability

of increasing the pH values in endo/lysosomes, due to the weaken red fluorescent signals (Figure S4 and S5). However, after repeating the experiments several times following the product operation protocols, we hardly quantitative analyzed the exactly pH value decreased among different cell line before or after feeding with BPSi NPs due to no correlation between intensity and the pH values established.

PDMPPO was then employed as a better solution for indicating the pH value changes after BPSi endocytosis, which introduce ratio imaging technics in pH quantitative measurement. PDMPPO [2-(4-pyridyl)-5-((4-(2-dimethylaminoethy- laminocarbamoyl) methoxy) phenyl) oxazole] is characterized as acidotropic dual-excitation and dual-emission pH probe. It emits intense green fluorescence at lower pH and gives intense blue fluorescence at higher pH. This unique pH-dependent fluorescence makes PDMPPO an ideal pH probe for acidic organelles with $pK_a = 4.47$. PDMPPO selectively labels acidic organelles (such as lysosomes) of live cells and the two distinct emission peaks can be used to monitor the pH fluctuations of live cells in ratio measurements. However, we still failed in measuring the pH values in 6 cell lines before and after BPSi feeding. As the results shown in Figure S6, no significant differences were observed in all 6 cell lines before and after BPSi feeding. Though a correlation has been established between the Blue/Green Ratio and the pH values (Figure S7), nonlinear correlation from pH 4-5 make the PDMPPO method failed in quantitative analysis of endo/lysosomes before and after BPSi feedings.

From the data of two commercial pH indicators above, we firstly demonstrated our suspect that PEG decorated nanoparticles with amide on the PEG chain could make the endo/lysosome pH increase due to alkaline nature of amide. However, without the quantitative analysis of precise pH changes (0.1 pH range), we still cannot establish the correlations between the autophagy status and the endo/lysosome pH values, thus failed in autophagy prediction.

Based on our previous study on self-decomposable SiO_2 nanoparticles, the MB dye grown in the center of the nanoparticles presented pH dependent release profiles. So, in this study, great efforts have been made to adjust the synthesize parameters, to make the MB release present linear correlation with the pH value changes. By adjusting MB and TEOS amount, we obtained 10 series self-decomposable nanoparticles with different sizes, MB loading efficiencies and drug release profiles, etc..

Based on our previous study on self-decomposable nanoparticles^[16-19], we kept the same concentration of Ammonium hydroxide in 75% ethanol, but adjusted the MB and TEOS concentrations. 2 series of TEOS amount have been set as 100 μ L and 80 μ L, in order to obtain different shell thickness and pore size. 10 series of MB amount have been set to obtain different size of center-hollow structure and MB loading efficacies. The MB and TEOS amounts added in the protocols were as described in the Table 1 below,

Table 1. MB and TEOS amounts added in the reaction solution

Groups	MB amount (mg)	TEOS amount (μL)
NPs _{1.0/100}	1.0	100
NPs _{1.5/100}	1.5	100
NPs _{3.0/100}	3.0	100
NPs _{4.0/100}	4.0	100
NPs _{6.0/100}	6.0	100
NPs _{1.5/80}	1.5	80
NPs _{2.0/80}	2.0	80
NPs _{2.5/80}	2.5	80
NPs _{5.0/80}	5.0	80
NPs _{7.5/80}	7.5	80

As shown in Figure 2a and 2b, the nanoparticles size increased with the increase of the MB amount, in both TEOS concentrations (100 μL and 80 μL). At the same MB concentration, the particle size increased with the increase of TEOS amount. Moreover, with the increase of the TEOS amount, the shell thickness growth up, which has been proved by the element mapping (shown as Figure 2c). The morphology studies predicted that with the increase of MB amount, the loading efficiency will grow up, leading to the faster release profile, while with the increase of TEOS amount, the release will be slow down. And we need to find out the appropriate MB and TEOS concentration, with which we could obtain the optimized nanoparticle systems, that we may be able to make the MB release profile linear correlated with the pH changes.

The MB loading efficiency was determined by UV-Vis spectrum. The standard curve (Figure S8) of MB was firstly drawn using series concentrations of MB solution (From 6.25 μg/mL to 46.88 μg/mL), with the equation as $y=67.63x+0.10919$, $R^2=0.9987$. As calculated with the equation above, we obtain MB loading efficiency of 10 self-decomposable nanoparticles with specific parameters, detailed data shown in Figure S9.

Before study the MB release profiles in different pH solutions, the release profiles in pure water have been studied. As shown in Figure S10 and Figure S11, all the nanoparticles with TEOS amount of 80 μL presented increased MB release along with the duration increase, which was reflected by the UV-Vis absorption. Moreover, with the MB encapsulated amount increase, the growth trend of MB release

become more significant. Also, the release velocity growth faster. However, as the TEOS amount increase to 100 μL , the particle surface became more dense and the release become slower when the MB amount below 3.0 mg, almost no increase trend could be observe in the MB release in water during 14 days release. As long as the MB amount increase to above 4.0 mg, obvious increase trend of MB release could be observed. One thing to be noticed is that the nanoparticles parameter of both NPs_{7.5/80} and NPs_{6.0/100} presented solid growth as the time prolong, almost showed linear increase trend during the first 7 days and then reached the platform.

Then we focused on the MB release behavior in different pH buffers to figure out whether self-decomposable nanoparticles with specific parameter could have the linear pH dependent MB release.

Firstly, we carried out the MB release experiments at pH 4.0 buffer solution. From Figure S12, we could easily reach the conclusion that with the same TEOS amount of 100 μL , the MB release velocity presented positive correlation with the MB encapsulated amount. Similar trend was observed in the 5 nanoparticle systems of TEOS at 80 μL (Figure S13). The center concentrated MB diffuse into the surrounding solution via diffusion due to concentration difference.

The bigger concentration gradient makes the faster MB release. Compared with the MB release in pure water, we found that the acidic environment speeded up the release of MB (Figure S12 and S13 compared with Figure S10 and S11), indicating that the MB release is not only driven by diffusion, however, in acidic solutions, electrostatic repulsion is also an important driven force due to the positive charge nature of MB. We then calculated the release percentage of each nanoparticle parameter according to the MB loading efficiency, MB standard curve and the dilution ratio at measurements. The release percentage reflected the release speed of MB in pH 4.0 acidic solution, and the results (Figure 3) showed that only the release percentage of NPs_{7.5/80} presented linear release in pH 4.0 solution. Other nanoparticle systems with specific MB and TEOS parameters though showed similar release trends, the release percentage did not linear growth. One exception is NPs_{6/100}, the MB release reached the platform in only 72 hrs, thus, it was hard to tell whether the MB release could growth linear before that duration at this stage.

Meanwhile, we tested the MB release profiles in near-neutral and alkali buffers (pH 6.86 and pH 9.18). The results in both Figure S14, S15 and Figure S16 and S17 demonstrated that the MB release slowed down with the solution pH increase to 6.86, moreover, with the central MB concentration increased, the MB release percentage decreased. At pH 9.18, all nanoparticles with 10 specific parameters presented very slow MB release (Figure S18 and S19)—no matter in UV-Vis absorption or the release percentage, the trend was similar with the one in pH 6.86 buffer, but with even lower release percentage. So, it was clear that the self-decomposable nanoparticles only presented MB release linear growth in acidic solutions. We thought back to the endo/lysosomes pH, from 4-5, which is exactly the pH range of MB linear growth as a function of time in specific MB/TEOS parameter. Thus, we get more confident that self-decomposable nanoparticle system maybe an accurate measuring tool for quantitative determining the endo/lysosome average pH, then provide evidence on the exactly pH value of autophagy status.

The precondition of using the specific self-decomposable nanoparticles as an endo/lysosome pH indicator is that the nanoparticles stay stable in the endo/lysosome during the whole measurement process. Secondly, the MB release in endo/lysosome should occur smoothly when the measurement carried out. The colocalization of the nanoparticle in the endo/lysosomes by cell TEM study and the MB release in 6 different cell lines were studied. From the cell TEM results, all of the 10 series nanoparticles stayed in the endo/lysosomes without escaping, after 24 hrs incubation with the HepG-2 cells (Figure 4).

We also investigated the intracellular location of the nanoparticles in other 5 cell lines, 4 nanoparticles were randomly selected to demonstrate the nanoparticles were trapped in the endo/lysosomes (Figure S20). The nanoparticles with all parameters showed central hollow structure in all other 5 cell lines after 24 hrs incubation, indicating the MB release. Moreover, under more precise observation, we noticed the MB release may be different due to different hollow sizes, pointing to the fact that 1. The endo/lysosomes pH in different cells are different, 2. The MB release from the nanoparticles is very sensitive to the endo/lysosomes pH, especially for the NPs $_{6/100}$ and NPs $_{7.5/80}$. From Figure S21 we can find that nanoparticles have already realized the endocytosis and stayed in the vesicles 2 hrs after nanoparticles feeding, then nanoparticles gradually accumulated in lysosomes.

We then evaluated the correlation between the pH values and the OD values in pH 4.0-4.8. From the results in Figure 5 and Figure S22, for NPs $_{6/100}$ and NPs $_{7.5/80}$ nanoparticle systems, the MB release presented linear decrease as a function of pH in the pH range from 4.0 to 4.8.

We then converted the OD value to MB release percentage according to the MB loading efficiency and feeding amount. As shown in Figure S23, in the first 6 hrs the MB release percentage in NPs $_{6/100}$ and NPs $_{7.5/80}$ nanoparticle systems also presented as a function of pH values. We then calculated the Residual Sum of Squares and Pearson's related coefficient at 6 hrs and 12 hrs release duration, respectively, as the Residual Sum of Squares present negative correlation with closeness of linear fitting, while the closer the absolute value of Pearson's related coefficient to 1, the more linear it is. As shown in Table S1 and S2, the highest degree of linearity is the fitting of NPs $_{6.0/100}$ nanoparticle systems, followed by the one of NPs $_{7.5/80}$ at 6 hrs release.

Till then, we were so excited by the results that the method for precisely monitoring the pH values has been established, especially with the accuracy less than or equal to 0.1 pH value interval. That means, we have great possibilities to figure out the correlation between endo/lysosome pH values and the autophagy status, which is of great significance for better studying the autophagy mechanism and predicting the autophagy process. As we can see in figure S24, the MB release in HepG-2 cells have already reach the plateau after incubation for 4 hrs. Thus, we chose the 6 hrs as the observation time point.

We then carefully investigated the MB release of NPs $_{6.0/100}$ in 6 cell lines in the nanoparticle cell interaction duration of 6 hrs, including liver cancer HepG-2 cell line, colon cancer HCT8, HCT 15 and HCT 116 cell lines, lung cancer A549 cell line and myomelanocytic cancer B16 cell line.

As shown in Table 2, we clearly differentiate the endo/lysosomes in 6 cancer cell lines, with the accuracy at 0.01 pH values, which is impossible to be done with the commercial intraocular pH indicator kits.

Table 2. Endo/lysosomes pH values calculated by equation established in 6 different cell lines

Cell lines	OD values of MB release	pH values calculated by equation $y=-0.07079x+0.40574$	Errors
HCT8	0.0656	4.806	0.077
HCT15	0.111	4.166	0.083
HCT116	0.081	4.593	0.065
HepG-2	0.072	4.708	0.088
A549	0.063	4.847	0.078
B16	0.062	4.855	0.092

Moreover, we re-evaluated pH values in endo/lysosomes of the HepG-2 cells before and after cultured with BPSi nanoparticles. We reached the conclusion that BPSi uptake significantly increase the endo/lysosome pH values, from 4.70 ± 0.09 to 5.59 ± 0.05 , perfectly illustrating the reason for BPSi uptaken induced the autophagy initially then terminated the autophagy flux. The intracellular uptake of BPSi make the quantities of the endo/lysosomes increased, which was consistent with the results of gene sequencing, that autophagy related genes (TFEB-CLEAR) was activated. Meanwhile, the autophagy termination by the increased pH values in endo/lysosomes was also coincide with the results of p62 proteins upregulation in Western Blot study.

4. Conclusion

In this work, we established a new method for endo/lysosomes pH qualitative determination based on self-decomposable nanoparticle systems. 10 nanoparticle systems with specific MB/TEOS parameters were employed for obtaining optimized pH sensitively responsive measurement method. The optimized central hollow nanoparticle system could release the central concentrated MB as a linear function of precise pH values in the range of pH 4.0-4.8, which is exactly the pH of lysosomes. Finally, by this qualitative pH indicator based on self-decomposable nanoparticles, we have succeeded in detection of the average pH values of lysosomes in 6 cell lines. Moreover, by this system, we can qualitative differentiate the pH changes of lysosomes before and after BPSi nanoparticles endocytosis by HepG-2 cells, clarifying the mechanism of the autophagy occurrence then termination after BPSi endocytosis. The self-decomposable nanoparticle systems pave a brand new way for studying the luminal pH values, providing new tools to know better of the cell signaling and metabolism.

Abbreviations

BPSi: Black mesoporous silicon

GO Gene ontology

FBS Fetal bovine serum

MB: Methylene blue

NPs: Nanoparticles

OD Optical Density

PBS Phosphate-buffered saline

PDMP: 2-(4-pyridyl)-5-((4-(2-dimethylaminoethyl) methoxy) phenyl) oxazole

KEGG Kyoto Encyclopedia of Genes and Genomes

RT-qPCR Reverse transcription quantitative polymerase chain reaction

TEOS: Tetraethyl orthosilicate

Declarations

Availability of Data

All data generated or analysed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by the National Science Foundation of China (No. 81771972), the National Key Research and Development Program of China (2017YFC0107405), and Innovation talents promotion plan of Shaanxi province (No. 2019KJXX-083).

Authors' contributions

LF and WCL conceived the idea and designed the project. CJS, YZL designed, synthesized and characterized MB@SiO₂ nanoparticles. CP and YZL carried out cell experiments for the BPSi nanoparticles. CP and CJS used HepG-2 cell line for cell sequencing, RT-qPCR and Western blot experiments. CP, YZL and QFW performed in vitro experiments, including MB release in NP in 4.0, 6.86 and 9.18 pH solutions, and cell uptake examination by TEM and confocal microscopic studies. XSZ, JWW, YT, HF, JWH cultured HepG2, HCT116, HCT15, HCT8 cell lines, and carried out lysosomal pH measurements by PDMP. CL, BLW culture A549 and B16 cells and related cell experiments. CP and CJS measured the lysosome pH with MB@SiO₂. CP and LF wrote the paper. All authors discussed the results.

All authors have approved the final version of the manuscript. CP, CJS and YZL contribute equally to this work.

Acknowledgements

Not applicable

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Figures

Figure 1

Schematic illustration of MB@SiO₂ measurement of lysosome pH in living cells.

Figure 2

Autophagy is activated in HepG-2 cells after feeding BPSi. (a) GO classification of the differentially expressed genes found by transcriptome sequencing, (b) GO enrichment bubble map of the differentially expressed genes found by transcriptome sequencing, (c) Verification of gene changes in the TFEB-CLEAR gene network after BPSi treated by RT-qPCT experiments, (d) P62, TFEB, LC3B β / α protein expression after BPSi treated, and (e) Normalized band gray intensity of P62, TFEB, LC3B β / α protein in BPSi treated groups according to the control group. Data was presented as mean $\pm$ S.D..

Figure 3

Morphology characterization of 10 different self-decomposable nanoparticles with specific MB or TEOS amount. (a) STEM figures, (b) Nanoparticles size distribution analysis and (c) Si mapping of 10 self-decomposable nanoparticles. Scale bars in all figures are 100 nm. The size distribution analysis was performed by randomly chosen 100 nanoparticles from STEM figures and measured by Image J software. Data was presented as mean $\pm$ S.D..

Figure 4

MB release percentage of 10 series self-decomposable nanoparticles after specific duration in pH 4.0 buffer. All experiments were triple repeated, and the data were shown as mean $\pm$ S.D.

Figure 5

The colocalization of the nanoparticle in the endo/lysosomes by cell TEM study after 12 and 24 hrs incubation with the 10 series nanoparticles

Figure 6

MB release as a function of pH values in NPs 6/100 and NPs 7.5/80 after specific incubation duration, 6 hrs, 12 hrs and 24 hrs. The linear correlation equations were also calculated for 6 hrs and 12 hrs for MB release from both NPs 6/100 and NPs 7.5/80 as a function of pH values. All experiments were carried out triplicated, and the data were shown as mean $\pm$ S.D..

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