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Real-Time Visualization of Lysosome Destruction Using a Photosensitive Toluidine Blue Nanogel

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Abstract

Breaking the lysosome helps its' sequestered payloads access their molecular targets in cells and thus enhances the intracellular drug delivery. Current strategies for lysosomal escape involve direct physical interactions with the lipid membrane. These interactions pose a systemic toxicity and uncontrolled membrane rupture risk. Here we report a light-detonated lysosome disruption using a hyaluronan (HA) nanogel packed with toluidine blue (TB). The HA/TB nanogel is concentrated within the lysosomes. The applied light assists TB in generating reactive oxygen species and destroying the lysosome in situ, both in cells and isolated lysosomes. Real time fluorescent tracking reveals that quenched TB fluorescence recovers along with lysosome explosion, re-locates to the nucleus, and is presented as a fluorescent sparkling in cells. This HA/TB, composed of all clinically approved materials, represents a biocompatible and facile strategy to “bomb” lysosomes in a spatiotemporally controlled fashion.

Graphical Abstract

Light triggered precision bombing of lysosome is achieved by toluidine blue (TB)-loaded hyaluronan (HA) nanogel (HA/TB). The detonation of the lysosome entrapped nanogel and release of lysosomal contents can be monitored in real time.

Keywords

Lysosome; Nanoparticles; Photodynamic therapy; ROS; Toluidine blue

Lysosomes, the intrinsic digestive system of cells, receive and sequester cargoes from phagocytosis, endocytosis and autophagocytosis.^[1] The lysosomal entrapment of drugs, therapeutic macromolecules, or nanoparticles is a natural barrier for delivery.^[2] The subsequent sequestration of drugs, especially those susceptible to degradation within the lysosomes, further hampers the drug's therapeutic efficacy, resulting in drug resistance.^[1b] Thus lysosome has been proposed as a target for cancer therapy. Breaking the lysosome could significantly improve drug delivery as well as the therapeutic efficacies. Current strategies that promote lysosomal escape include lysosome destabilization and destruction.^[2a,3] Lytic agents, such as chloroquine, polycationic molecules, cell penetrating peptides and pore-forming molecules, have been applied to destabilize lysosomes through osmotic swelling or direct interaction with the lipid membrane.^[4] The photo-destruction strategy, such as photochemical internalization, provides better temporal and spatial control based on light generated reactive oxygen species (ROS).^[3,5] To obtain a maximal photo-destruction effect, photosensitizers (PS) require a strict co-localization within the membrane lipids because of the limited radius of action (10–20 nm) and short half-life (~ 20 ns) of singlet oxygen.^[4c,6] Very recently, a liquid-metal nanoparticle, which consisted of gallium/indium alloy and graphene quantum dots, was developed to promote lysosomal escape.^[7] The nanoparticle efficiently converts photoenergy to heat and generates ROS to disrupt the membrane.

Learning from prior arts, it was hypothesized that a simple PS enriched nanoparticle under a similar photo-controlled manner could be an effective bomb for lysosome destruction. To demonstrate the concept, toluidine blue (TB) and hyaluronan (HA) were used to prepare the light sensitive nanobomb. HA is a natural linear polysaccharide with a targeting capability towards CD44, a surface receptor highly expressed on many cancer cells. Its presence assures a concentrated localization within the lysosomes once the HA based nanogels are internalized through CD44-mediated endocytosis.^[8] For over 50 years clinicians have used TB as mouth rinse to highlight the oral lesions.^[9] It is also an excellent photosensitizer evidenced by its current use in multiple clinical trials of antibacterial photodynamic therapy (PDT).^[10] TB has demonstrated a strong singlet oxygen quantum yield and a favored absorption (633 nm) peaked in the far-red window (600–900 nm).^[11] The ideal cationic-aromatic structure of TB makes it able to be noncovalently packaged into HA to form a HA/TB nanogel using a recently developed protocol.^[8c] The quenched TB fluorescence in HA/TB aids in the convenient monitoring of the intracellular release of TB. During its release, the particle becomes de-quenched and enables the real-time tracking of the light triggered lysosome explosion.

To prepare the HA/TB nanogel (Figure 1a), an aqueous solution of HA, TB and a temporary crosslinker, cisplatin, was heated at 90°C for 1 hour. The solution was then cooled on ice and subjected to exhausted dialysis in PBS. While in PBS, cisplatin, the reversible crosslinker, was spontaneously removed from the nanogel, while it retained TB within the nanogel. The acquired HA/TB appeared as homogenous spheres with a diameter of 55.2 nm determined by a transmission electron microscopy (TEM) (Figure 1b). In water, HA/TB had a negative charge of –28.4 mV and a hydrodynamic size of 264.3 nm from the dynamic light scattering (DLS) analysis (Table S1). Around 62.5% of TB was successfully loaded into the nanogel while only 1.24 % of cisplatin was retained (Table S1). The low levels of cisplatin in the

nanogel supported the successful removal of the crosslinker (cisplatin), as we have previously demonstrated.^[8c]

Compared to free TB, the TB within the nanogel demonstrated a broadened blue-shift absorption and a near complete fluorescent quench (Figure 1c–d); thus implying that the aggregation of TB in the nanogel is due to its' π -conjugated planar structure, which favors molecular stacking when the local TB concentration is high.^[12] The aggregated TB within the nanogel led to a weaker ability to generate singlet oxygen molecules under LED light (633 nm) when compared to free TB (Figure 1e). The reduction of photosensitivity could be compensated when TB is released and accumulate within the lysosome following the degradation of HA.^[8c,13] This lysosome-assisted release of TB was successfully mimicked by monitoring the TB's fluorescent recovery in HA/TB when it was incubated with a pH 5 buffer containing hyaluronidase (HAase) (Figure S1), the two key features (low pH and enzymes) that account for lysosomal degradation. However, HA/TB was stable in a PBS and bovine plasma solution, proven by the appearance of a main peak in the hydrodynamic size distribution, well maintained after 3 days at 37°C (Figure S2).^[14] More importantly, no burst release of TB was found during incubation. The total TB released was minimal in both PBS and plasma (7.68% and 17.72% at day 3, Figure 1f). This stably dispersed HA/TB in plasma guarantees that the majority of TB will be picked up by the cells while in particle form.

To verify the intracellular localization of HA/TB, an MDA-MB-231 breast cancer cell line was chosen due to its high expression of CD44.^[8b,8c,15] CD44 is responsible for the specific uptake of HA and its' derivatives. Following incubation, free TB at 10 μ M produced a nonspecific stain of the whole cell, especially the nucleus (Figure S3b). In contrast, the HA/TB signals were strictly confined within the lysosomes even when the TB concentration was raised from 10 to 80 μ M (Figure 2a and S3), highlighting the critical role of the nanogel in modulating the distribution of TB within the cells. TB, itself, is able to cross the membrane, freely diffuse into the cytoplasm and bind DNA in the nucleus.^[9] Interestingly, the use of free TB (10 μ M) compromised the stain observed from the LysoTracker (Figure S3b), which was commonly noted in a panel of cationic molecules.^[16] Most targeted nanoparticles are internalized through a receptor mediated endocytosis and trafficked in the endo-lysosomal system.^[17] The packing of TB within the nanogel guided it to the lysosomes due to the HA/TB's nano-size and CD44 targeting natures. The compact structure of HA/TB may have also contributed to this concentrated distribution due to the slow extracellular release of TB, as observed from the drug release experiment (Figure 1f). It was noticed that the fluorescent intensity of HA/TB in cells was much weaker than that of free TB due to the quenched fluorescence of TB in the nanogel. Nevertheless, this quenched fluorescence makes tracking TB upon lysosomal escape possible in real time. The released TB would quickly gain fluorescent signals that are easy to track and observe.

Consequently, cells loaded with HA/TB were continuously excited by LED light (95m W/cm² at 633 nm) and recorded for 3 minutes with a fluorescence microscope. The TB signal within the cells first decreased due to a photo-bleaching effect. The dot-like signals then started to sparkle, spread and was then redistributed to the cell's nucleus (Figure 2b and Movie S1). Our video clip could be the first real time presentation of a nanoparticle-enabled lysosome “bombing”, and the leakage of lysosomal contents.

To confirm that the fluorescent sparkling was associated with lysosome leakage, an acridine orange (AO) staining method was used.^[18] Under normal culture conditions, AO collected within the lysosome was seen as vacuoles within the cell. However, when the lysosome was damaged by light induction aided by HA/TB, AO leaked into the cytoplasm and entered the nucleus as seen in Figure 3a–b. Similar fluorescence and nuclear translocation after PDT have been documented for other PS, such as phthalocyanines.^[19]

Furthermore, cells were pre-incubated with a quantum dot based Qtracker and used as a lysosome tracker.^[20] The Qtracker has a strong anti-photobleaching ability, and renders a constant fluorescent signal for vesicle tracking after LED irradiation, as was demonstrated in the control cells (Figure S4a). When the cells were incubated with HA/TB, irradiated and compared with unexposed cells, more Qtracker signals were observed to have diffused into the cytoplasm, indicating the leakage of lysosomes (Figure S4b). The similar fluorescent diffusion and increase in the cytoplasm after lysosome destabilization has also been documented by Gillmeister et al using GFP as fluorescent indicator.^[21]

To prove direct lysosomal disruption, the lysosome fraction of HA/TB treated cells was isolated. The loading of HA/TB in harvested lysosomes could be seen by fluorescent microscope (Movie S2). Under continuous irradiation, the fluorescent sparkling phenomenon was also observed in the isolated lysosomes. An examination of the ultrastructure of the lysosomes affirmed the lysosome's disruption. Compared with unilluminated lysosomes, rough and disrupted lysosomal membrane structures were spotted after light exposure (Figure 3c–d). While the ultrastructure of control lysosomes remained intact with the same light irradiation challenge (Figure S5). These evidences strongly suggested that HA/TB restrained in lysosomes could be detonated by light to blow up the lysosome membrane.

The fluorescence of TB in cells was quantified by flow cytometry (FACS) at different time points of light exposure with the aim to probe the underlying mechanism of the HA/TB mediated lysosome disruption. In line with the generation of fluorescence in the Movie S1, the fluorescence of HA/TB incubated cells was gradually increased following light irradiation (Figure 4a), while the untreated cells did not show any fluorescent change (Figure S6). ROS, the main product of PDT, was one of the common factors that destabilize the lysosome's membranes due to the oxidation of its lipids.^[22] Consequently the intracellular ROS mediated by HA/TB was evaluated using DCFDA staining. As shown in Figure 4b and S7, HA/TB treated cells had a rising ROS level positively related to the LED irradiation time, while the controls, including the LED illuminated cells, and HA/TB treated cells, only demonstrated a minimal ROS production. It was noted that the TB fluorescence increase was lagging behind the generation of ROS in cells. After 30 seconds of irradiation, the TB fluorescence started rising, while the intracellular ROS rate was already 6.2 folds over cells that had no light exposure (Figure S8). This strongly suggested that ROS, initialized by the HA/TB or together with its' released TB within the lysosome, caused the lysosomal rupture which set the trapped TB free. The lysosome's disruption would induce the release of its contents, such as cathepsins, which further triggers the cell death cascade.^[1a,22b] The continuous LED exposure killed cells in a time-dependent manner as visualized by the live/dead cell staining (Figure 4c). This accounted for the different degree of lysosome damage

controlled by irradiation time. Since HA is proven to be highly biocompatible and degradable,^[8a] the observed phototoxicity was assigned to the TB within the nanogel. In fact, free TB has a clear toxicity towards MDA-MB-231 cells with an IC₅₀ of 1.99 μ M in darkness (Figure S9b), presumably due to its strong ability to bind nucleus which may cause mutagenesis.^[23] By confining TB in lysosomes, HA/TB exhibited a significantly reduced toxicity in darkness, IC₅₀ dark = 128.3 μ M (Figure S9c). Importantly, the light stimulated HA/TB to destroy the lysosomes and turned the storage depots into distribution hubs by sending the restrained TB to the nucleus; thus, offering a toxicity of a low μ M range, IC₅₀ light = 8.1 μ M. In terms of PDT efficiency, the light-dark toxicity ratio was improved from 3.2 for free TB to 15.8 for HA/TB (Figure S9), speaking for the benefits of the current nanogel in bombing lysosomes.

In summary, the current study provides a facile, biocompatible and spatiotemporally controlled approach to break the lysosome using two clinically used materials HA and TB (Scheme 1). When TB is stably packed in HA/TB, it can be specifically transported to the lysosomes by the nanocarrier, avoiding the nonspecific distribution of free TB in cells. Furthermore, the LED light is able to stimulate the nanogel to generate ROS and act as a “precision bomb” to disrupt the lysosome. The real time fluorescent tracking enabled by HA/TB reveals that the escaped TB quickly binds the nucleus, which might further enhance the cytotoxicity. Generally, future applications of this lysosome bomb would lie on the cargoes loaded in the nanogel. With the assistance of this HA nanogel, other PS could be specifically ferried into lysosome to perform lysosome-based PDT. When a drug is co-loaded with HA/TB, this lysosome ‘bombing’ effect might also be extended to the photochemical internalization application to increase the drug delivery efficiency.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Figure 1.

Characterization of HA/TB nanogel. a) Schematic illustration of HA, TB and HA/TB nanogel. b) Morphology of HA/TB observed by TEM. c) UV-Vis-NIR absorption spectra of TB and HA/TB in water. d) Fluorescent spectra of TB and HA/TB. TB concentration was fixed at 10 $\mu\text{g/ml}$. e) Light induced singlet oxygen generation determined by singlet oxygen sensor green (SOSG). f) Drug release of HA/TB in contact with PBS and bovine plasma at 37°C.

Figure 2. Intracellular localization of HA/TB in MDA-MB-231 cells. a) Co-localization of HA/TB with lysosome and nucleus in darkness. b) Intracellular distribution of TB throughout the LED light exposure (Movie S1). Cells were treated by HA/TB (80 μM TB) for 20 hours.

Figure 3.

Light triggered destabilization of lysosome. a-b) Acridine orange (AO) staining of the control (a) and HA-TB treated cells (b) with or without light irradiation. The red and dark vacuoles in cells suggest the intact lysosomes. c-d) TEM images of isolated lysosomes from the HA/TB treated cells. The damaged ultrastructure was only seen after light illumination.

Figure 4. Dynamic changes of HA/TB treated cells following the light illumination. a) Typical TB fluorescence of cells. b) Intracellular ROS generation. c) Live (calcein AM) and dead (PI) staining of cells. The white dash line defines the area exposed to LED light.

Scheme 1.

Schematic illustration of HA/TB in precision lysosome bombing.