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Invited Review

Tracing nanoparticles and photosensitizing molecules at transmission electron microscopy by diaminobenzidine photo-oxidation

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ABSTRACT

During the last three decades, diaminobenzidine photo-oxidation has been applied in a variety of studies to correlate light and electron microscopy. Actually, when a fluorophore is excited by light, it can induce the oxidation of diaminobenzidine into an electron-dense osmiophilic product, which precipitates in close proximity to the fluorophore, thereby allowing its ultrastructural detection. This method has very recently been developed for two innovative applications: tracking the fate of fluorescently labeled nanoparticles in single cells, and detecting the subcellular location of photo-active molecules suitable for photodynamic therapy. These studies established that the cytochemical procedures exploiting diaminobenzidine photo-oxidation represent a reliable tool for detecting, inside the cells, with high sensitivity fluorescing molecules. These procedures are trustworthy even if the fluorescing molecules are present in very low amounts, either inside membrane-bounded organelles, or at the surface of the plasma membrane, or free in the cytosol. In particular, diaminobenzidine photo-oxidation allowed elucidating the mechanisms responsible for nanoparticles internalization in neuronal cells and for their escape from lysosomal degradation. As for the photo-active molecules, their subcellular distribution at the ultrastructural level provided direct evidence for the lethal multiorganelle photo-damage occurring after cell photo-sensitization. In addition, DAB photo-oxidized samples are suitable for the ultrastructural detection of organelle-specific molecules by post-embedding gold immunolabeling.

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1. Introduction

With its pioneering paper, Maranto (1982) first demonstrated that diaminobenzidine (DAB) and light illumination might be used to convert the fluorescence of intracellularly injected Lucifer Yellow into a signal suitable for examination in bright-field microscopy. Based on this article, other authors then provided evidence that

Abbreviations: DAB, diaminobenzidine; FS, fluorogenic substrate; NP, nanoparticle; PS, photosensitizer.

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a wide variety of fluorochromes with specificity for different substances can be DAB photo-oxidized into reaction products, which can thus be visualized at both light and electron microscopy (see for instance, Sandell and Masland, 1988; Lubke, 1993; Singleton and Casagrande, 1996).

Photo-oxidation has been originally proposed as a procedure to transform the unstable fluorescent signal into a stable product; afterward, it has become the technique of choice for detecting the location of fluorescent molecules at the high spatial resolution provided by transmission electron microscopy. Thus, photo-oxidation of fluorescent markers has been widely used to investigate the nervous tissue, above all to trace neuronal networks (Balercia et al., 1992; Buhl, 1993; Gan et al., 1999; Hanani et al., 1999) and analyze synaptic vesicle turnover (Harata et al., 2001; LoGiudice et al., 2009; Meunier et al., 2010; Welzel et al., 2011; Hoopmann et al., 2012).

The application of refined techniques of electron tomography on photo-oxidized samples allowed the 3D reconstruction of different organelles and structures, such as microtubules, the endoplasmic reticulum and the Golgi apparatus (Ladinsky et al., 1994; Meisslitzer-Ruppitsch et al., 2008); and to elucidate dynamic processes such as endocytosis (Fomina et al., 2003; Liu et al., 2005; Lichtenstein et al., 2009; Kukulski et al., 2011; Schikorski, 2010; Röhrl et al., 2012) and exocytosis (Kishimoto et al., 2005; Liu et al., 2005).

Photo-conversion of fluorochrome-labeled lipids or proteins was used to investigate the 3D organization and interaction of the membranous compartments at the junction between the trans-Golgi network and the endoplasmic reticulum (Pagano et al., 1989; Meisslitzer-Ruppitsch et al., 2008). Hence, confirming earlier immunocytochemical data at a higher spatial resolution, and providing novel evidence for the subcellular localization and dynamics of specific molecular components of the Golgi membranes (Meisslitzer-Ruppitsch et al., 2008).

Mainly in more recent years, photo-oxidation has been used for the direct ultrastructural visualization of the green fluorescent protein (Grabenbauer et al., 2005), and has also been associated with techniques of fluorescence recovery after photobleaching (FRAP) (Darcy et al., 2006), or adapted for confocal laser scanning microscopy (Colello et al., 2012).

The high sensitivity of photo-oxidation makes it a reliable method to detect in situ fluorescent molecules precisely, even when they are present in low amounts. Indeed, the photo-oxidation reaction, giving rise to the electron-dense products, depends on the free oxygen radicals that originate from the fluorochrome irradiation and leads to the local oxidation of DAB: the networks of oxidized DAB appear in the form of finely granular precipitates whose contrast may be enhanced by osmium staining. Due to the very short half-life (from 1 ns to 1 μs) of oxidizing chemical species – such as singlet oxygen, the hydroxyl radical or the superoxide radical – their spatial mobility is limited to 1–30 nm only (Karuppanapandian et al., 2011). Consequently, precipitation of oxidized DAB occurs very closely to the real place where reactive oxygen species are produced upon light irradiation of naturally fluorescent or fluorescently labeled samples.

In recent times, this peculiar feature has been exploited for two original applications of photo-oxidation procedures in transmission electron microscopy: tracking the fate of fluorescently labeled nanoparticles in single cells, and detecting the subcellular location of photo-active molecules suitable for photodynamic therapy.

1.1. Nanoparticles in biomedicine

Nanoparticles (NPs) are receiving great attention in the diagnostic and therapeutic fields as biocompatible carriers for tracing molecules or drugs. Polymeric NPs are especially recognized as useful drug delivery systems, since they can cross capillaries and enter the cells, allowing an efficient drug accumulation at the targeted sites where they are able to prolong drug activity by stabilizing the encapsulated drugs and modulating their release (Béduneau et al., 2007; Mundargi et al., 2008). This allows sustained drug release over a period of days or even weeks after administration (Jallouli et al., 2007). NPs have also recently been proposed as suitable tools for theranostics, i.e. the integrated therapy and diagnostics for personalized medicine (Kim et al., 2013).

Currently, chitosan is one of the most investigated polymers in the field of pharmaceutics: in fact, chitosan is biocompatible and biodegradable, it exhibits relatively low immunogenicity and toxicity, and it may be easily functionalized and complexed (Kumar et al., 2004; Freier et al., 2005; Rinaudo, 2006). The polycationic nature of chitosan enables interactions with the cell membranes; under some experimental conditions, chitosan-based NPs have shown to cross biological barriers (Schipper et al., 1997; Peppas and Huang, 2004), including the blood brain barrier (Karatas et al., 2009; Songjiang and Lixiang, 2009; Wang et al., 2010; Jaruszewski et al., 2012). In addition, chitosan NPs proved to escape endosomes after cell internalization, thus protecting the incorporated drugs from lysosomal degradation (Koping-Hoggard et al., 2004; Douglas and Tabrizian, 2005; Serda et al., 2010; Zaki et al., 2011). It is worth noting that chitosan-based NPs have shown to be capable of entering the cell nucleus and delivering active molecules, at least in HeLa cells (Colonna et al., 2011).

Based on these features, chitosan NPs have been envisaged by our group as promising drug delivery systems for targeting hypometabolizing opioids of potential biomedical interest to the central nervous system (Malatesta et al., 2007). There is an obvious interest in biomedicine for inducing a hypometabolic state with a consequent decrease in body temperature; e.g., this could advance the surgical procedures that imply hypothermy (such as in aortic arch surgery: Yan et al., 2013), or help to preserve organs for transplantation (Tisherman, 2013), or to improve neuro- and cardio-protection under ischemic conditions (Arrich et al., 2012).

Chitosan NPs have already been demonstrated to efficiently load and release the synthetic hypometabolizing delta opioid D-Ala(2)-D-Leu(5)-enkephalin, inducing a hypometabolic state in cultured epithelial cells (Colonna et al., 2011). However, designing a drug delivery strategy requires preliminary studies on target cells so as to clarify: (i) NP uptake mechanisms and timing; (ii) the intracellular fate of internalized NPs and their relationships with cell organelles; and (iii) the possible structural and functional alterations related to the intracellular permanence of NPs. In particular, the intracellular degradation pathway of NPs is crucial for estimating their efficacy as drug carriers: in fact, upon entry by endocytosis, endosome-entrapped NPs generally fuse with the acidic lysosomes, resulting in sequestration and degradation of the loaded molecules by the lysosomal enzymes (Panyam et al., 2002).

The endocytotic uptake of chitosan NPs has been previously studied by conjugating the polysaccharide with fluorescent dyes (Nam et al., 2009; Chiu et al., 2010; Zaki et al., 2011). Unfortunately, the elucidation of the exact intracellular trafficking pathway of NPs is difficult, due to the dynamics of maturation and traffic between the endosomal and lysosomal compartments (Watson et al., 2005). Transmission electron microscopy allows visualizing chitosan NPs at high resolution in the intracellular space; however, the homogeneous and moderate electron density of chitosan NPs makes them almost indistinguishable from the cytosolic milieu, thus limiting relevant information. DAB photo-oxidation applied to fluorescently labeled chitosan NPs proved to represent a valuable approach to overcome this limitation.

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