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Mini review article

POLYOXOMETALATES: A NEW CLASS OF STAINING AGENTS

POLIOKSOMETALATI: NOVA KLASA JEDINJENJA ZA BOJENJE

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Abstract

For decades, transmission electron microscopy (TEM) has been one of the most valuable methods for analysing the ultrastructure of biological specimens, especially in the research field of medicine. Samples for TEM need quality preparation to obtain a contrast that can be detected in electron micrographs. Substances that make it possible to achieve a quality contrast in TEM are salts of heavy metals. One of the most successful substances for achieving contrast in TEM is uranyl acetate (UAc), because of its highly detectable contrast properties. Nevertheless, the latest change in regulations about the radioactivity of materials gravely prohibits the use of UAc, although it would be applied exclusively for scientific reasons. Considering that UAc is dangerous because of both its toxicity and radioactivity, the buildout of a new secure substitute is essential. This is the present problem in the TEM procedure which urges to be solved. This review aims to show the probability of use of polyoxometalates (POMs) as new potential contrast agents. Polyoxometalates are heavy atom sources with a wide range of biological activities. Extensive chemical characterization of POMs brings promising use of these substances as new contrast agents for TEM observation of biological samples.

Keywords:
transmission electron microscopy,
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Sažetak

Transmisiona elektronska mikroskopija (TEM) već decenijama predstavlja jednu od najvrednijih metoda za ultrastrukturnu analizu bioloških uzoraka, posebno u oblasti medicinskih istraživanja. Potrebna je kvalitetna priprema bioloških uzoraka za TEM kako bi se dobio kontrast koji se može detektovati na elektronskim fotomikrografijama. Supstance koje omogućavaju postizanje kvalitetnog kontrasta za TEM jesu soli teških metala. Jedna od supstanci koja najuspešnije postiže kontrast u TEM proceduri je uranil-acetat (UAc), zbog veoma uočljivih kontrastnih svojstava. Ipak, najnovija izmena propisa o radioaktivnosti materijala zabranjuje upotrebu UAc, čak i u čisto naučne svrhe. S obzirom na to da je UAc opasan zbog svoje toksičnosti i radioaktivnosti, neophodan je razvoj nove bezbedne zamene. Ovo je aktuelan problem koji postoji u proceduri za TEM i zahteva hitno rešenje. Cilj ovog preglednog članka je da prikaže mogućnosti primene polioksometalata (POM) kao novih potencijalnih supstanci za kontrastiranje TEM uzoraka. To su teški atomski izvori sa širokim spektrom bioloških aktivnosti. Opsežna hemijska karakterizacija POM-ova obećava potencijalnu upotrebu ovih supstanci kao novih agensa za kontrastiranje bioloških uzoraka za TEM.

Ključne reči:
transmisiona elektronska mikroskopija, polioksometalati, uranil-acetat, sredstva za kontrastiranje

Introduction

Transmission electron microscopy (TEM) has long been the principal method for ultrastructural characterization of biological samples in medical research (1). A crucial advantage of the transmission electron microscope is its resolution, accomplished by using the electron beams. Beams of electrons use high voltage for acceleration, which gives them worthwhile wavelengths, the ones shorter than the wavelength of visible light (1). Practically, in real-life conditions, some circumstances can have an influence on resolution (1). In this review, a brief history of the development of TEM, and the principle of its work will be shown,with sample preparation for TEM as an introduction to new problems that this significant method deals with nowadays. The emphasis is on the urgent problem in TEM procedure, that appear due to contemporary limitations in the use of uranyl acetate (UAc), one of the oldest contrast agents previously used in TEM (2). Among other substances that have been recently tried as a replacement for UAc, one of the noteworthy candidates for contrast that could be used for TEM are polyoxometalates (POMs). Polyoxometalates are clusters of metal ions with a negative charge and a high oxidation state.

This review aims to gather all information published about POMs until present day and explain why they could be potentially promising new contrasting substances for routine staining of ultrathin sections that are used for TEM observation of structures.

Brief history of TEM

The very beginning of the electron microscope (EM) dates to the 1920s (1). It can be stated that the main reason for the development of EM was the restricted resolution of light microscope photographs (1). In the mid-1920's, Louis de Broglie was the first scientist to come up with the idea that the electron has characteristics of a wave, with a wavelength shorter than that of visible light (1). Two years later, Davisson and Germer and Thomson and Reid, two completely independent research teams, performed experiments that showed the wave-like features of electrons (1). These revelations helped future scientists to come up with the idea of constructing a new instrument – the electron microscope.

Among the earliest discoveries in this field was a patent made by Rüdenberg in 1931 (3). The main flaw of Rüdenberg's research was that he described the development of EM only theoretically, but did not present any experiment to confirm his theory. Meanwhile, in United States, Davisson & Calbick illustrated the principle of forming an electron image and determined the optical constant of the lens (4). Crucial year for the development of EM was 1932, when Max Knoll, alongside Ruska and von Borries, his students, constructed the instrument that was able to form the image thanks to the electron beams (5,6). Knoll and Ruska published the paper about their discoveries, in which they named one chapter "*Das Elektronenmikroskop*" and that is when the term EM was first introduced in literature (6). This discovery was a most crucial step in the development of EM, for which Ruska received the Nobel Prize. The power of Ruska's breakthrough was seen through the fact that the commercial transmission electron microscope (TEM) was first developed only four years later (1). Another important name in the world of electron microscopy is Ladislaus Laszlo Marton. Marton constructed a transmission electron microscope to observe the nature of electrons in Brussels in 1932 (7). Over the next two years, a few new electron microscopes were developed, mostly used in the field of industry (7). First implementations of TEM outside the industrial framework started in 1934, and that is also the year when Marton´s microscope found its use in biological sciences (1,7). All these discoveries inspired engineers and scientists to construct more efficient instruments for TEM observations and to work on new developments and discoveries that will potentially improve the use of TEMs.

Tissue preparation for TEM

The transmission electron microscope is a supreme instrument for analysis of the ultrastructure of the cells; however, for that to happen, it is necessary to prepare the sample in the best possible way. Due to electron radiation damage that can affect the sample, good tissue preparation is a precondition for the TEM procedure (8). Sample preparation for TEM consists of fixation, dehydration, infiltration, embedding and contrasting (9,10). Safeguarding the environment and equipment is needed to reduce the harmfulness of the procedure. When the fresh tissue is collected during the experiment or the autopsy, it needs to be immediately put in a fixative solution for TEM (10). The sample should be cut into 1mm^3 size pieces and maintained at 4 °C in a vial of primary TEM fixative, glutaraldehyde (10). Aldehydes are used as fixatives for chemical fixation to cross-link proteins. Next step is the osmium post fixation stage, which needs to be done to complete the stabilization of the specimen (10). Osmium tetroxide attaches to the lipid components of membranes, and that is why it is used to infiltrate the cells and make them more rigid. Therefore, osmium tetroxide is used both for the fixation of membranes in cells and as a contrasting agent that makes membranes more visible (10). After fixation, samples are washed in distilled water, after which the water should be removed. Next step is placing the water with an organic solvent that is non-volatile in the vacuum of the microscope, such as ethanol and propylene oxide (1,10). Epoxy resins are thermosetting polymers of choice for embedding tissues for TEM (10). After the process of polymerizing is done, the block of epoxy resins is prepared for the next step, sectioning (10). The thickness of the samples for the TEM procedure varies from 50 to 500 nm, depending on the needs of the research (10). An ultramicrotome is an instrument for sectioning the specimen (11). Ultrathin sections are cut using a diamond knife, after which they are placed on the metal grids (usually copper) (10,11).

Staining for TEM – urgent problem that must be solved

Uranyl acetate (UAc), alongside lead citrate, is the main duo utilized for the contrasting of the tissue for the TEM procedure (2). The combination of these two substances gives a quality contrast of structures, which results in good black and white ultraphotomicrographs (2,12). The double staining protocol with UAc and lead citrate has been commonly used as the most successful staining protocol for TEM for decades (12,13). The structure of the uranyl acetate is shown in **Figure 1**.

Even though UAc has been used as a staining agent for TEM for years, its use in the laboratory was always under strict policies and rules (14-16). Uranyl acetate is toxic when inhaled or ingested, as uranium is a heavy metal and can damage the kidneys. Ingestion and especially inhalation of uranium compounds can lead to an increased risk of cancer. Salts of uranium can irritate skin, eyes, and mucosae of many different organs (14-16). Its hazardous and toxic potential has more and more consequences on human life. Also, the struggle with the disposition and secure repository of UAc made this problem worse, even for already established microscopy laboratories (17). Acquiring even a small quantity of this reagent represented a big problem for researchers and technicians, because the procedure of getting the authorization from the government was very long, slow and ponderous. Even modern, well-organized research centres did not consider the welfare of this very quality reagent worth all that work (17).

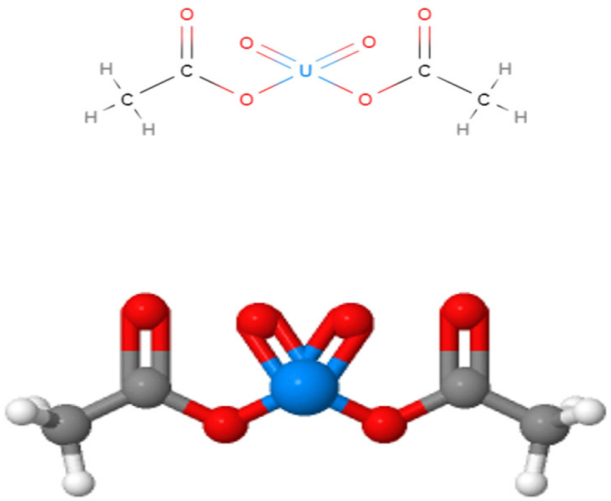

Figure 1. Uranyl acetate structure – molecular formula (up) (U- uranium, O-oxygen, C-carbon, H-hydrogen) and ball and stick model (down) of (blue-uranium, red-oxygen, grey-carbon, white-hydrogen)

New regulations put these salts into the category of nuclear fuels and forbid their use (especially in Europe and Japan), even for purely scientific purposes (17). A couple of researchers in Japan tried to find a solution for this problem. They tested some hafnium salts, traditional Chinese tea, and platinum blue colour as staining agents for EM, and some of them are now commercially available (18-20). Unfortunately, these substances were not stable in the solutions, so managing them was quite difficult (18-20). French company Chromalys, alongside Delta Microscopes, commercialized a new stain for TEM under the name ”Uranyless” (free from uranyl). This stain is created from lanthanide salts and other substances stable to stain tissue at pH around 6.5 (21). However, new staining agents that are presently used instead of UAc do not show as high contrast as UAc, even though it was shown that they do not disrupt cell integrity (18-21). All previously mentioned facts and changes in regulations regarding radioactive hazards indicate that finding a new and secure substitution for UAc is a serious necessity in the world of TEM research. This big scientific and health problem should be solved as soon as possible.

Negative Staining for TEM with Heavy Metals

Besides its use as a staining agent for TEM, UAc has also been widely used as a negative staining reagent for observation of some macromolecules in the TEM procedure

(22). Ever since van Bruggen and collaborators presented UAc as a very potent contrast reagent, many other salts of uranium were examined and approved for use for the same purpose (23). The story of negative staining for TEM begins in mid 1950s, when the main problem in specimen observation in the TEM procedure was the complication of differentiation of the structure of interest from other structures in the immediate vicinity. Cecil Hall was one of the first researchers to investigate different staining methods for viruses and published the results of his work in 1955 (24). In this study, he tried to stain a particle of virus with phosphotungstic acid (PTA), but the experiment did not have positive outcomes. On the contrary, it showed that the application of PTA to viral particles gave a negative contrast effect (24). Next year, in 1956, Hugh Huxley came to the same conclusions with his study about tobacco mosaic virus (25). The significance of the negative staining method in TEM observations of viral and other macromolecular particles was first completely explained by Sidney Brenner and Robert Horne in 1959 (26). The basis of the negative staining technique lies in the use of heavy metal in the stain solution, thanks to which, when the solution dries, a thin, dense layer around the structure of interest is formed. The structure has a lower atomic mass than the solution with heavy metal and that results in black and white contrast on ultraphotomicrographs taken on TEM (26). This technique allows us to visualize structures smaller than 100 nm, like viruses, molecules of enzymes, and even some cell compounds like microtubules and ribosomes. Another advantage of this procedure is that small particles do not need to pass through the sectioning procedure and can be directly visualized under the TEM (10). In the 1990s, researchers carried on with the investigation of negative staining methods and came up with the conclusion that this procedure could be applied to samples smaller than 1nm, using frozen section techniques (26, 27). Although there are numerous papers about the application of this technique in biological sciences, the use of the negative staining method in material sciences has not show that much success. During all these years, only several papers were published, with reinforcement tests of other heavy metal solutions as new possible reagents for the negative staining technique (17). Lanthanides were the first new group of chemical elements to be tested for this purpose. For these experiments, first they tried lanthanum, as the main representative of the group, and then gadolinium and samarium (17). The first to show the application of lanthanide salt in the negative staining technique was Bradley in the 1960s. He tested lanthanum triacetate in solution with thorium nitrate or thorium chloride and detected visible contrast on ultraphotomicrographs (28). It was not until the 1980s that lanthanum salts were tested again as staining agents, only this time for other reasons. Leeson and Higgs tested lanthanum trichloride for what they called intracellular staining (29). Other lanthanide salts like acetates of gadolinium and samarium have shown stability in solutions and prove to have pretty good negative staining characteristics (2).

Polyoxometalates – new possible staining agents

Polyoxometalates (POMs) are clusters of metal ions with a negative charge and a high oxidation state, such as molybdenum, tungsten and vanadium (30). Main representatives of the polyoxometalates' family are shown in **Table 1.**

Table 1. Most known members of the POMs family

Structure	Formula
The hexametalate structure	$[M_6O_{19}]^{x-}$
The Keggin structure	$[XW_{12}O_{40}]^{x-}$
The Wells-Dawson structure	$[X_2W_{18}O_{62}]^{x-}$
The Pope-Jeannin-Preyssler structure	$MP_5W_{30}O_{110}]^{x-}$
The trivacant Keggin-derived sandwich complex	$[(M^{II})_2(M^{II}L)_2(PW_9O_{34})2]^{16-}$
The trivacant Wells-Dawson-derived sandwich complex	$[(M^{II})_2(M^{II}L)_2(PW_{15}O_{56})^2]^{16-}$
The double-Keggin structure	$[\{A\text{-}\alpha\text{-}SiO_4W_9O_{30}(OH)_3M_3\}^2]^{11-}$
HPA-23	$[NaSb_9W_{21}O_{86}]^{18-}$

x-the charge, M-central metal ion, O-oxygen, X-heteroatom, W-tungsten, P-phosphorus, Si-silicon, Na-sodium, Sb-antimony, HPA-antimonium tungstate

Polyoxometalates have been of interest in many fields, such as nanotechnology and electronics (31). Their antitumor, antidiabetic and antimicrobial functions have been recently examined (30, 32-34). In the last couple of years, there have been research papers explaining the potential of POMs as good contrast agents for computed tomography (CT) (35-38). They have a very large compositional variety and are potentially applicable in many fields thanks to their specific properties. These substances are thermally stable, soluble in solutions and have fascinating characteristics regarding catalysis, magnetism and photo- and electrochemistry (30, 39).

It was mentioned earlier in the paper that PTA is one of the first staining agents used for negative staining of viruses (22, 24). Keggin-type phosphotungstate is mostly used PTA from this group and its structure consists of molecules of tungsten, phosphorus and oxygen, connected into one functional very acidic cluster (40). Keggin-type PTA belongs to group of polyoxometalates, more precisely their class called polyoxotungstates. Polyoxotungstates are clusters of tungsten and oxygen molecules, with anionic characteristics (30, 41, 42). They are stable molecules, usually used in solutions with NaOH or KOH, salts that neutralize their dominantly acid parts (42).

However, there is the research that shows that ultraphotomicrographs of samples that were stained with Keggin-type PTA do not show as good and quality contrast as those ultraphotomicrographs of samples that were

stained with UAc (40). These research results motivated scientists to start new studies about good substitutes for negative staining reagents for TEM. Different members of phosphotungstates´ family were tested as new potent substitutes for staining reagents for TEM. Some experiments examined phosphotungstates´ family members, sodium silicotungstate and methylamine tungstate, as new possible staining reagents for negative staining for TEM, but results remained the same, as in the previous studies with different compounds of this family (41, 42). One of latest papers from 2022 presented Preyssler-type phosphotungstate as new quality reagent for negative staining procedure for TEM (40). This study showed that Preyssler-type phosphotungstate potassium salts give detectable contrast in TEM ultraphotomicrographs of viruses (40). In this experiment, researchers also made the comparison between UAc, Keggin- and Preyssler type PTA to analyse which of these substances gives the best detectable contrast in TEM observation of viruses (40). Preyssler-type phosphotungstates shows neutral characteristics and does not need to be neutralized *a priori*, while Keggin-type phosphotungstates and UAc are primarily acidic molecules, that need neutralization. Results of this later study show that Preyssler-type phosphotungstates may be good and quality new substitutes for negative staining reagents for TEM observations of viral particles (40).

All latter mentioned discoveries about polyoxotungstates and lanthanides as substitute for UAc as negative staining reagents open new possibilities for applying POMs as staining reagents, not only for negative staining of viruses, but staining of all biological tissues for TEM observations. It is highly possible that other classes of POMs can be also used as new staining agents for TEM, which is yet to be discovered.

Conclusion and future prospects

Transmission electron microscopy has made a revolution in the world of microscopy and enabled us to see the complete ultrastructure of materials and tissues. The resolution of TEM allows us to see the structure up to the level of atoms and particles. With every day, new improvements and advanced options of this instrument are discovered, regarding nanotechnology, crystallography and many other fields. Still, limitations of TEM come up from the fact that the sample must be perfectly prepared. One of the main problems nowadays is staining and contrasting the sample, because, it is safe to say, now forbidden use of UAc, regarding its radioactive, toxic and hazardous characteristics. This problem needs to be solved. Recent studies investigated the potential role of some classes of POMs as contrast agents for the negative staining for TEM, which is collected here in review. Nature and scientific application of POMs have been examined for decades, but only a few new reports examined POMs as new probable substitutes for the negative staining procedure for TEM. Considering their bioavailability, compatibility, biostability and especially their non-radioactivity and non-toxicity, new possibilities for the use of POMs use are opened, not only for the existing ones, but also for new synthesized POMs. Let's not forget to mention that relative cost efficiency in POMs synthesis, compared to other contrast agents, is just one more of their advantages.

Summarizing all previously mentioned information, the conclusion of this review is that the investigation of the use of POMs as contrast agents could be a huge step in the development of new staining and contrasting methods for TEM. Finding a good substitute for hazardous and radioactive UAc could possibly be done, and this urgent scientific problem could find its solution in POMs. All information gathered in this review leaves a lot of space for researchers to explore this topic and maybe synthesize some new POMs suitable as staining and contrasting agents for TEM observations.

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