Compartmentalized Intracellular Click Chemistry with Biodegradable Polymersomes

RESEARCH ARTICLE

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Compartmentalized Intracellular Click Chemistry with Biodegradable Polymersomes

Roy A. J. F. Oerlemans, Jingxin Shao, Sander G. A. M. Huisman, Yudong Li, Loai K. E. A. Abdelmohsen, and Jan C. M. van Hest**

Polymersome nanoreactors that can be employed as artificial organelles have gained much interest over the past decades. Such systems often include biological catalysts (i.e., enzymes) so that they can undertake chemical reactions in cellulo. Examples of nanoreactor artificial organelles that acquire metal catalysts in their structure are limited, and their application in living cells remains fairly restricted. In part, this shortfall is due to difficulties associated with constructing systems that maintain their stability in vitro, let alone the toxicity they impose on cells. This study demonstrates a biodegradable and biocompatible polymersome nanoreactor platform, which can be applied as an artificial organelle in living cells. The ability of the artificial organelles to covalently and non-covalently incorporate tris(triazolylmethyl)amine-Cu(I) complexes in their membrane is shown. Such artificial organelles are capable of effectively catalyzing a copper-catalyzed azide-alkyne cycloaddition intracellularly, without compromising the cells' integrity. The platform represents a step forward in the application of polymersome-based nanoreactors as artificial organelles.

1. Introduction

Polymeric vesicles, or polymersomes, have over the past years found widespread application as nanoreactors.[1] Since the pioneering work of Meier and coworkers, a range of different polymersomes with semipermeable shells have been constructed, which allow the transport of small molecules across the polymer membrane.[2–10] By encapsulation of biocatalysts in the aqueous lumen of the polymersomes, these enzymes can still effectively convert their substrates, while being protected from external factors such as proteases or mechanical stress. The robustness of the polymersome shell facilitates recyclability of the catalysts, for example in flow processes.[11] Furthermore, it provides opportunities for coencapsulation of multiple enzymes to effectively carry out cascade processes.[5,12] These specific features also facilitate the exploitation of polymersomes as nanoreactors in cellulo or even in vivo.[6,9,10,13–18]

So far, most reported polymersome nanoreactors employ enzymes as catalytic species, which are traditionally located in the polymersome lumen. However, the polymeric membrane does not only provide protection and controlled permeability, but also a hydrophobic environment. The presence of such a hydrophobic shell can be used for the sequestration of more hydrophobic substrates, which are then more easily converted in aqueous media. This concept has been extensively investigated in the field of polymer micelles, for example by Lipshutz and O'Reilly.[19–23] Intriguingly, such catalyst-loaded micelles were even applied in mice for the activation of prodrugs.[24,25] In the field of polymersomes, the number of examples of membrane anchored catalysts remains remarkably low. To the best of our knowledge, there are currently only two reported examples; both of which involve polymersomes prepared from poly(ethylene glycol)-*b*-polystyrene (PEG-*b*-PS) either loaded with an organic catalyst or an organometal complex. Aqueous asymmetric aldol reactions were catalyzed by polymersomes with covalently bound L-proline in the membrane.[26] Immobilization of a bis-oxazoline Cu(I) complex in the polymersome membrane resulted in nanoreactors that promoted aqueous asymmetric cyclopropanation reactions.[27]

We are particularly interested in the Cu(I)-tris(triazolylmethyl)amine complex, as this catalyst is abundantly used for copper-catalyzed azide-alkyne cycloaddition (CuAAC) reactions. After the discovery of the first ligand, tris(benzyltriazolylmethyl)amine (TBTA),[28] many new variants have been developed, resulting in increased reaction kinetics, improved water-solubility and biocompatibility.[29–31] Moreover, CuAAC reactions promoted by such complexes have been applied on the surface of living cells as well as intracellularly.[32] In most cases, free catalysts have been used for these reactions.

R. A. J. F. Oerlemans, J. Shao, S. G. A. M. Huisman, Y. Li, L. K. E. A. Abdelmohsen, J. C. M. van Hest
Department of Bio-medical engineering and Chemical engineering & Chemistry
Eindhoven University of Technology: Technische Universiteit Eindhoven
P.O. Box 513, 5600 MB Eindhoven, The Netherlands
E-mail: l.k.e.a.abdelmohsen@tue.nl; j.c.m.v.hest@tue.nl; j.vanhest@science.ru.nl

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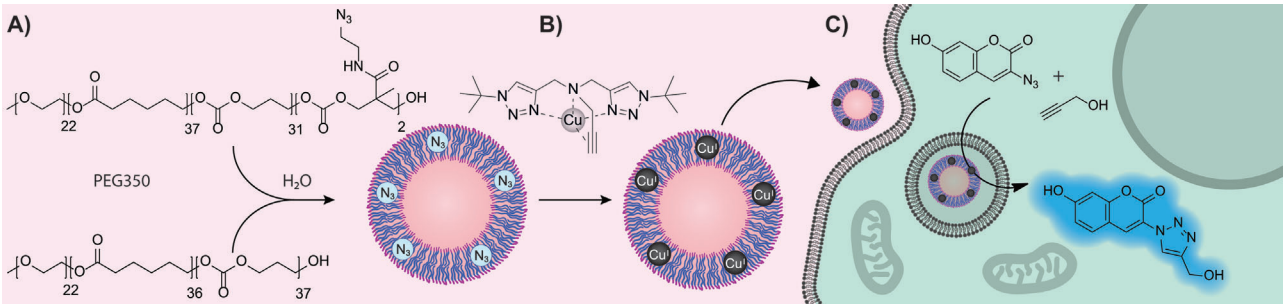

Figure 1. Schematic overview of the preparation and intracellular activity of polymersomes with a covalently loaded copper catalyst. A) A mixture of two copolymers self-assembles into polymersomes with azides located in the hydrophobic domain. B) The polymersomes are covalently loaded with a copper catalyst via chelation-assisted CuAAC. C) Cellular uptake of the nanoreactors is followed by intracellular CuAAC to form a fluorescent product as an efficient read-out.

Nevertheless, exposure of cells to high concentrations of transition metals can lead to toxicity. To overcome this limitation, various nanocarriers have been developed to transport transition metals inside living cells, shielding the catalyst whilst minimizing unwanted contact with the intracellular environment. Besides being potentially less invasive, nanocarriers furthermore offer more control over the localization of the catalyst. However, examples reported of such copper-loaded nanoparticles have their drawbacks. They require in situ activation of the catalyst by either reduction or oxidation and, [33–36] additionally, most of the reported nanocarrier systems lack biodegradability, which is disadvantageous as an accumulation of intracellular waste could lead to apoptosis.[37] Therefore, in this work, we set out to immobilize the tris(triazolylmethyl)amine-Cu(I) catalyst complex in biodegradable polymersomes, constructing a nanoreactor platform that can be safely integrated within living cells (**Figure 1**).

Polymersomes built from block copolymer poly(ethylene glycol)-*block*-poly(caprolactone-*gradient*-trimethylene carbonate) (PEG-P(CL-*g*-TMC) present an ideal platform to host a catalyst in their membrane. Such polymersomes are inherently semipermeable due to the flexible nature of the membrane, which allows for catalyst loading and passive transport of small molecules.[9] The activity of such nanoreactors, therefore, does not rely strongly on the hydrophobicity of the substrates, in contrast to polymersomes prepared from PEG-*b*-PS. Furthermore, the block copolymer is composed of repeating ester and carbonate bonds, making the system prone to hydrolysis and therefore biodegradable. Accordingly, we designed PEG-P(CL-*g*-TMC) polymersomes with azides in the hydrophobic domain, which were used for the covalent attachment of a Cu(I)-tris(triazolylmethyl)amine catalyst. In parallel, polymersomes were loaded with copper catalyst complex in a non-covalent manner and the catalytic activities of both types of nanoreactor were compared. Finally, the nanoreactors were introduced to the complex environment of a living cell to examine their performance as artificial organelles.

2. Results and Discussion

Polymersomes were prepared by co-assembly of two different block co-polymers: PEG-P(CL-*g*-TMC) and its azide-functionalized counterpart. The former polymer was prepared by cationic ring-opening polymerization of caprolactone and trimethylene carbonate (TMC), using monomethoxy-poly(ethylene glycol) (1kDa) as macroinitiator and methanesulfonic acid as a catalyst, as reported previously.[9,38] The latter polymer was prepared in a similar fashion, with the incorporation of an additional monomer, a TMC with an azide-functionalized side chain. Polymersomes were assembled by hydrating a 5 wt% copolymer solution of a mixture of 80 wt% PEG-P(CL-*g*-TMC) and 20 wt% of azide-functionalized polymer in oligo(ethylene glycol) via direct addition of 4 volume equivalents of water.

In order to immobilize Cu(I) into the polymersome membrane, we made use of chelation-assisted copper(I) catalyzed azide-alkyne cycloaddition. In this approach, Cu(I) is first complexed to a ligand bearing one of the two functional groups required for CuAAC. For this purpose, the tris(triazolylmethyl)amine ligand was modified by replacing one of its arms with a propargyl moiety to generate bis(*tert*-butyl-triazolylmethyl)propargylamine (BTTPA). By employing a substrate that was at the same time a ligand for Cu(I) it was previously demonstrated that this resulted in dramatically increased reaction kinetics, even in biorelevant media.[39,40] Upon "clicking" to the polymersomes' azides in the membrane using the complex' own Cu(I) center as catalyst, a third triazole was formed, thereby achieving both catalyst immobilization and completion of the tris(triazolylmethyl)amine ligand. Noteworthy mentioning is that the self-assembly of copolymers functionalized with BTTPA resulted in a mixture of morphologies and therefore we set out to first prepare the polymersomes and subsequently load them with the catalyst. Hereafter, the polymersomes were extensively washed to remove any unbound copper. The polymersomes were characterized by cryogenic transmission electron microscopy (cryo-TEM) and dynamic light scattering (DLS), revealing an average size of 161 nm with a polydispersity index of 0.15 (**Figure 2**A,B). Successful coupling of the copper complex was confirmed by proton nuclear magnetic resonance (^{1}H NMR) spectroscopy, as well as inductively coupled plasma mass spectrometry (ICP-MS) analysis. In the ^{1}H NMR spectrum, the signal of the methylene adjacent to the azide shifted upon conjugation of the catalyst complex from its original position at 3.45 ppm. In addition, a new peak arose at a chemical shift of 8.09 ppm, representing the triazole protons (Figure 2C). ICP-MS analysis indicated that a copper loading of 75.7 ± 1.6% was achieved, relative to full functionalization, that is 44 nanomoles copper per milligram of polymersomes.

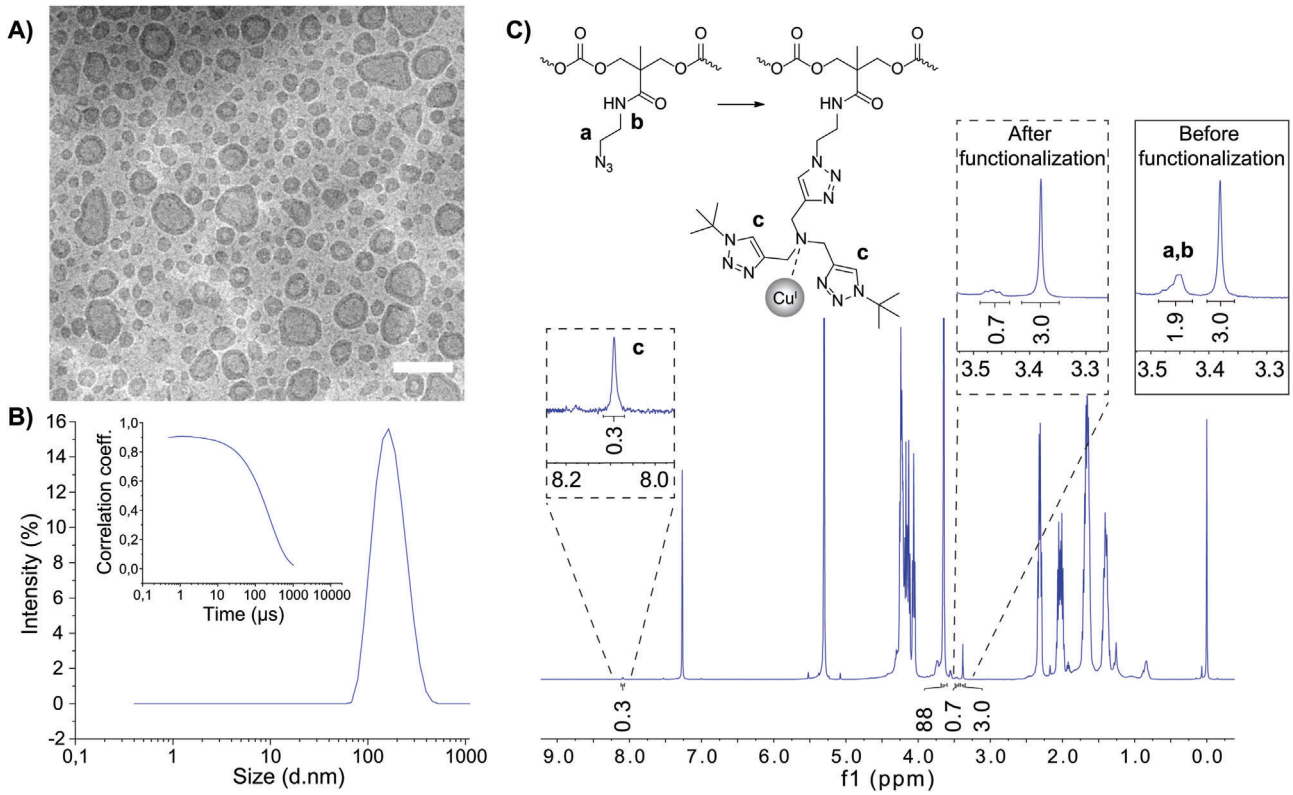

Figure 2. Characterization of polymersome nanoreactors with covalently loaded copper catalyst. A) Cryo-TEM image of the nanoreactors. Scalebar = 200 nm. B) mean DLS intensity profile and correlogram of the nanoreactors ($n = 3$). C) ^{1}H NMR spectroscopy analysis of the nanoreactors after catalyst loading, showing a change compared to non-loaded polymersomes, confirming successful functionalization.

Besides covalent catalyst loading, also a non-covalent embedment of the complex within the membrane was investigated. Two hydrophobic tris(triazolylmethyl)amine ligands were used for this purpose, namely tris(*tert*-butyltriazolylmethyl)amine (TTTA) and tris(benzyltriazoylmethyl)amine (TBTA). Polymersomes were treated with Cu(I) complexes prepared from these ligands and subsequently washed by spin filtration. These polymersomes were prepared using only PEG-P(CL-*g*-TMC) to prevent the presence of non-reacted membranal azides, which could be undesired reaction partners in an activity assay with external alkynes. According to ICP-MS analysis, a copper loading of 16.9 ± 0.1 nmol mg^{-1} polymer and 2.38 ± 0.03 nmol mg^{-1} polymer was achieved, for TBTA and TTTA, respectively (**Figure 3**C). This corresponds to a loading efficiency of 21.1% for TBTA-Cu(I) and 3.0% for TTTA-Cu(I). By covalent loading of the catalyst, copper retention after extensive washings was significantly higher compared to the non-covalent systems, demonstrating the advantage of the first approach. The higher copper loading of TBTA with respect to TTTA can be attributed to its increased hydrophobicity.

Subsequently, the catalytic activities of the covalent and the two non-covalent types of nanoreactors were examined by reacting the fluorogenic substrate 3-azido-7-hydroxycoumarin with propargyl alcohol (Figure 3A). The reactions were carried out under normal air atmosphere in presence of L-ascorbic acid, using a nanoreactor concentration of 4 mg mL^{-1}. As expected from the amount of copper loading, covalently-loaded nanoreactors as well as non-covalently loaded TBTA-nanoreactors showed the best performances based on fluorescence, reaching 90.7 ± 3.2% and full conversion, respectively (Figure 3B). The yield of the TTTA-nanoreactor-catalyzed reaction was only 14.1 ± 1.5%, which can be attributed to the low catalyst loading of these nanoreactors. When a lower amount of 1 mg mL^{-1} of the covalently loaded nanoreactors was used, the reaction still reached 96.4% ± 3.4% conversion. As tris(triazolylmethyl)amine ligands are known to stabilize the Cu(I) oxidation state of the catalyst, we set out to examine our nanoreactors' ability to promote the CuAAC reaction in the absence of a reducing agent. To this end, we omitted L-ascorbic acid from the reaction, and investigated the catalytic conversions. Interestingly, the covalent Cu(I)-nanoreactors still promoted CuAAC with a yield of 74.3 ± 3.9% and performed better than the TBTA-nanoreactors, which led to only 40.6 ± 1.8% conversion (Figure S30, Supporting Information). Moreover, when 3-azido-7-hydroxycoumarin and propargyl alcohol were converted via CuAAC, the catalytic nanoreactors displayed faster reaction kinetics compared to the corresponding free catalyst, tris(hydroxypropyltriazolylmethyl)amine-Cu(I) (THPTA-Cu(I)), using the same catalyst concentration (Figure S32, Supporting Information). For all catalyst concentrations studied, the initial slope of fluorescence generation was steeper in the case of the nanoreactors, compared to free catalyst. The lower concentrations of THPTA-Cu(I), 17, and 34 μM were not even able to convert all of the substrate to the corresponding fluorescent product. Possibly, the free catalyst was more prone to

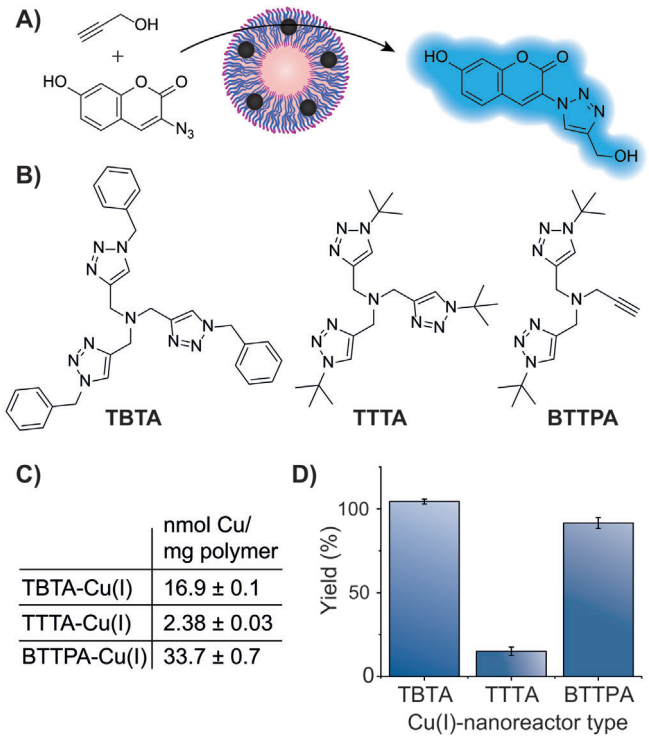

Figure 3. Nanoreactor performance. A) Reaction scheme of the CuAAC between 3-azido-7-hydroxycoumarin and propargyl alcohol. B) Chemical structures of TBTA, TTTA, and BTTPA. C) Copper content of the different types of nanoreactors. D) Catalytic activity of nanoreactors prepared from TBTA-Cu(I), TTTA-Cu(I), and BTTPA-Cu(I). The latter one is covalently attached to the membrane.

oxidation compared to the encapsulated catalyst, despite the presence of L-ascorbate.

In order to demonstrate the potential of our nanoreactors as artificial organelles, they were added to HeLa cells to allow for cellular internalization. Nanoreactors with covalent catalyst in their membrane were used, as they showed the best overall performance. The nanoreactors' cytotoxicity was evaluated first by treating the cells with varying concentrations of polymersomes (up to 1.0 mg mL^{-1}) for 16 h, followed by an MTT assay. At all nanoreactor concentrations, the cells retained their viability, with almost no sign of cytotoxicity (Figure S33, Supporting Information). Having demonstrated the biocompatibility of our nanoreactors, we examined whether the internalized nanoreactors were still catalytically active. Starting with the 16 h incubation of HeLa cells with fluorescently labeled nanoreactors (BODIPY FL), the cells were washed to remove any noninternalized nanoparticles. Then the fresh medium was added, supplemented with substrates 3-azido-7-hydroxycoumarin and propargyl alcohol. After 2 h, the cells were washed again and imaged by confocal laser scanning microscopy (CLSM) to visualize the formation of fluorescent product. In contrast to cells treated with polymersomes lacking copper, the nanoreactor-treated cells showed a clear fluorescent signal related to product formation by CuAAC, demonstrating the intracellular catalytic activity of the nanoreactors (**Figure 4**).

3. Conclusion

In summary, we have developed a biodegradable nanoreactor platform based on PEG-P(CL-*g*-TMC) polymersomes, in which a discrete Cu(I)-tris(triazolylmethyl)amine complex is covalently bound in the membrane at a post-assembly stage. The successful integration of the complex in the membrane was confirmed, and

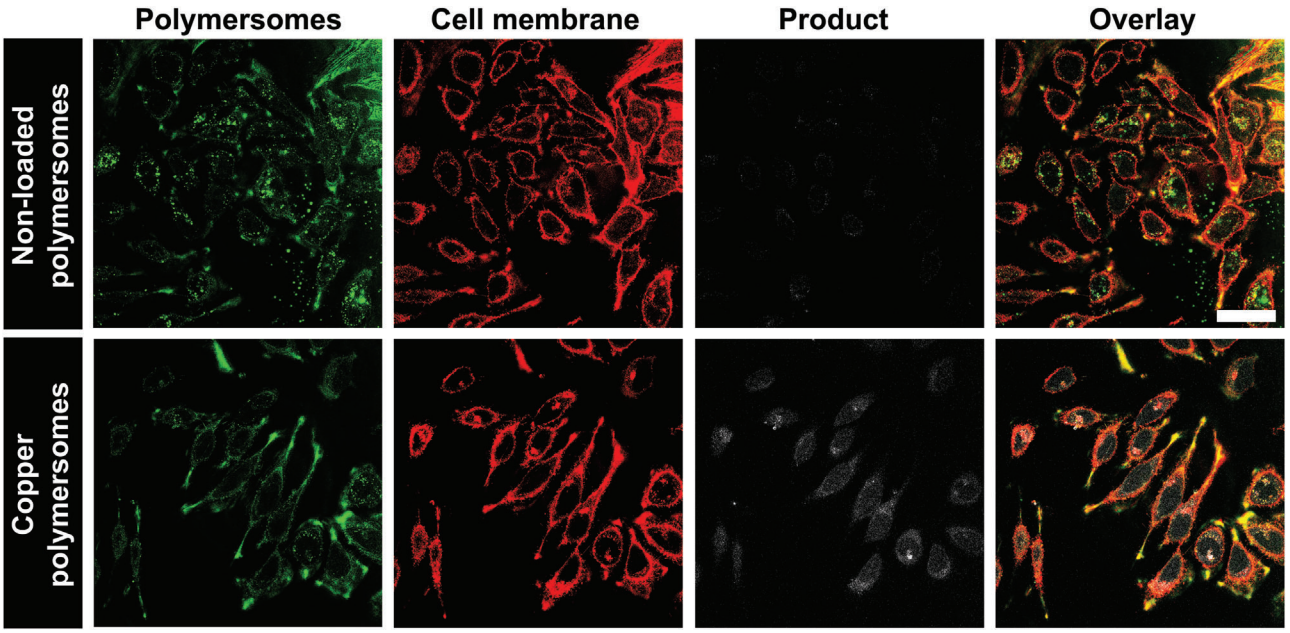

Figure 4. Confocal laser scanning microscopy images of HeLa cells treated with either non-loaded polymersomes (upper row) or catalytic polymersomes (lower row), followed by treatment with 3-azido-7-hydroxycoumarin and propargyl alcohol. Scalebar = 50 µm.

resulted in a higher copper loading and overall better catalytic performance compared to polymersomes which were allowed to take up hydrophobic copper complexes in a non-covalent manner. Our nanoreactors are biocompatible, and their incubation for 16 h with cells, at concentrations as high as 1.0 mg mL^{-1}, did not impact the cells' viability. Finally, we demonstrated the application of our nanoreactors as artificial organelles by showing the ability to catalyze CuAAC reactions in cellulo.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

artificial organelles, click chemistry, intracellular catalysis, metal catalysis, polymersomes

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