THE SILESIAN UNIVERSITY OF TECHNOLOGY

FACULTY OF CHEMISTRY

DEPARTMENT OF ORGANIC CHEMISTRY, BIOORGANIC CHEMISTRY AND BIOTECHNOLOGY

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EXTENDED ABSTRACT OF

DOCTORAL DISSERTATION

The collection of published and thematically related articles

Trehalose releasing nanogels for autophagy stimulation

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Supporting supervisor: dr inż. Małgorzata Milewska

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Table of content

List of publications

This Doctoral dissertation consists of the following collection of published and thematically related articles:

[**P1**] Maruf A*, Milewska M*, Varga M, Wandzik I*. *Trehalose-Bearing Carriers to Target Impaired Autophagy and Protein Aggregation Diseases*.
J. Med. Chem. 2023, 66, 15613−15628. DOI: 10.1021/acs.jmedchem.3c01442.
(IF_{2022} = 7.300, MEiN = 200 point, *contributed equally)

[**P2**] Maruf A, Milewska M, Lalik A, Wandzik I. *pH and Reduction Dual-Responsive Nanogels as Smart Nanocarriers to Resist Doxorubicin Aggregation*. **Molecules**. 2022, 27, 5983. DOI: 10.3390/molecules27185983.
(IF_{2021} = 4.927, MEiN = 140 point)

[**P3**] Maruf A, Milewska M, Lalik A, Student S, Wandzik I. *A Simple Synthesis of Reduction-Responsive Acrylamide-Type Nanogels for miRNA Delivery*. **Molecules**. 2023, 28, 761. DOI: 10.3390/molecules28020761.
(IF_{2022} = 4.600, MEiN = 140 point)

[**P4**] Maruf A, Milewska M, Kovács T, Varga M, Vellai T, Lalik A, Student S, Borges O, Wandzik I. *Trehalose-releasing nanogels: A step toward a trehalose delivery vehicle for autophagy stimulation*. **Biomater. Adv.** 2022, 138, 212969. DOI: 10.1016/j.bioadv.2022.212969.
(formerly known as **Mater. Sci. Eng. C** with IF_{2021} = 7.328, MEiN = 140 point)

[**P5**] Zhong Y*, Maruf A*, Qu K, Milewska M, Wandzik I, Mou N, Cao Y, Wu W. *Nanogels with covalently bound and releasable trehalose for autophagy stimulation in atherosclerosis*. **J. Nanobiotechnol**. 2023, 21, 472. DOI: 10.1186/s12951-023-02248-9.
(IF_{2022} = 10.200, MEiN = 140 point, *contributed equally)

Total IF = 34.355, Average IF = 6.871, Total MEiN = 760 point

Patent application

Ilona Wandzik, Małgorzata Milewska, Ali Maruf; Sposób otrzymywania nanożeli uwalniających kowalencyjnie związaną trehalozę.
Patent application number: P.438753 , submission date: 16 August 2021, Urząd Patentowy RP, Warszawa, Poland.

Summary of own contribution

I have significantly contributed to five publications ([**P1**], [**P2**], [**P3**], [**P4**], and [**P5**]) through various roles, ranging from experimental work to manuscript preparation and revision. Below is a brief summary of my contributions:

- Publication [**P1**] – 32% contribution. I participated in literature searching, manuscript writing, figure creation, managing copyright permissions, citation management, formatting, assisting in peer review revisions, and final proofreading.
- Publication [**P2**] – 65% contribution. I conducted experiments on anionic pH/reduction dual responsive nanogels, drug release studies, DLS and zeta potential measurements, cryo-TEM sample preparation, and cytotoxicity assessment. Additionally, I was responsible for data analysis, manuscript writing, figure and table creation, citation management, formatting, assisting in peer review revisions, and final proofreading.
- Publication [**P3**] – 60% contribution. I performed experiments on cationic reduction-responsive nanogels, miRNA release studies, DLS and zeta potential measurements, colloidal stability investigation, cryoTEM sample preparation, fluorescein-labelled nanogel synthesis, cell uptake for confocal imaging, and cytotoxicity assessment. I also handled data analysis, manuscript writing, figure and table creation, citation management, formatting, assisting in peer review revisions, and final proofreading.
- Publication [**P4**] – 30% contribution. I synthesized trehalose-releasing nanogels, conducted release and enzymatic assays, DLS and zeta potential measurements, colloidal stability investigation, cryoTEM sample preparation, fluorescein-labelled nanogel synthesis, cell uptake studies, and hemocompatibility assays. I participated in data analysis, manuscript writing, figure and table creation, citation management, formatting, assisting in peer review revisions, and final proofreading.
- Publication [**P5**] – 30% contribution. I contributed to the conceptualization of the study on trehalose-releasing nanogels for atherosclerosis treatment, synthesized nanogels, conducted release and enzymatic assays, DLS and zeta potential measurements, colloidal stability investigation, cryoTEM sample preparation, and Cy5-labelled nanogel synthesis. I also handled data analysis, manuscript writing, figure creation (including BioRender), citation management, formatting, assisting in peer review revisions, and final proofreading.

1. Research aims and the scope of the study

This doctoral dissertation is a collection of five published articles: one literature review presenting the current state of the research in the field of trehalose carriers targeting impaired autophagy, and four research articles focused on the synthesis, characterization, and the application of trehalose-releasing nanogels as potential trehalose carriers for autophagy stimulation. The collection of articles is supplemented with patent application. The primary aim of the work was to address the issue of poor trehalose bioavailability by developing covalent, yet hydrolytically-labile (releasable) incorporation of trehalose into nanogels to create nanogels that can release trehalose at systemic pH. Another aim was to evaluate capability of the developed trehalose-releasing nanogels to stimulate autophagy.

The scope of the study included:

- Elaboration of the synthesis of nanogels *via* photoinitiated free radical polymerization in inverse miniemulsion to establish optimal conditions for synthesis of trehalose-releasing nanogels.
- Study on the influence of the composition of trehalose-releasing nanogels on their physicochemical characteristics. The physicochemical characterization included: confirmation of covalent trehalose incorporation (by ^{1}H NMR), imaging (by cryoTEM), determination of trehalose content (by enzymatic assay), determination of hydrodynamic diameter (d_H) and zeta (ζ)-potential (by DLS and ELS, respectively), and evaluation of colloidal stability in selected biological media (by turbidimetry and DLS).
- Study on trehalose release from nanogels focusing on how it is influenced by nanogels composition as well as nanogels concentration and solution pH. The following compositional aspects were considered: content and structure of acrylamide-type units, type of trehalose ester and type of ionic functionalization.
- Fluorescent labeling of nanogels for bioimaging purposes by two different approaches. The first approach involved introducing the fluorescent moiety during polymerization, while the second approach involved incorporating it after polymerization.
- Evaluation of cytocompatibility and hemocompatibility of trehalose-releasing nanogels.
- Investigation on autophagy inducing potential of trehalose-releasing nanogels in *in vitro* and *in vivo* study in collaboration with Dr. Máté Varga's group from Eötvös Loránd University (ELTE), Hungary and Prof. Wei Wu's group from Chongqing University, China.

2. Introduction to research field: trehalose-containing nanocarriers to target impaired autophagy

Trehalose is a non-reducing homodisaccharide composed of two D-glucose units linked at their anomeric positions by an α,α'−1,1'−glycosidic bond. It is one of the most important disaccharides due to its high stability (resistance to high temperature and hydrolysis) and unique properties, *e.g.*: cryo-protection, protein stabilization, and autophagy induction.

Autophagy induction property of trehalose and its application in disease treatments is very promising and interesting topic to study [1], [2], [3]. Autophagy is a cellular recycling process that degrades cytoplasmic components, including damaged organelles, protein aggregates, and lipid droplets and recycles them *via* a degradative organelle–lysosome in response to a variety of stress stimuli (*e.g.*, nutrient and energy stress, hypoxia, and pathogen invasion) [4], [5]. This process is important to maintain cells homeostasis, differentiation, and survival. Due to its critical role in cellular processes, the imbalance and dysfunction of autophagy are connected to a number of human disorders, such as neurodegenerative disorders, cardiovascular disease, pulmonary disorders, renal diseases, cancer, autoimmune disorders, and metabolic syndromes [4], [5].

The clinical trials of trehalose for the treatments of diseases related to impaired autophagy and protein aggregation has been summarized in [**P1**] [6]. There are currently 9 ongoing clinical trials using trehalose administered orally or intravenously for the treatments of type 2 diabetes, Parkinson's disease, Alzheimer's disease, acute coronary syndrome, amyotrophic lateral sclerosis, and spinocerebellar ataxia type 3 according to the data from the Clinical trials database (https://clinicaltrials.gov). One major drawback, however, is that to maintain its ability to induce autophagy, trehalose must be administered at extremely high doses due to its hydrophilic nature and susceptibility to enzymatic degradation [2]. The examples are:100 mM for *in vitro* study and 2–3 g/kg/day (administered parenterally) or 2–4 w/v (administered orally in drinking water) for *in vivo* study. Additionally, a report in Nature, which was published in 2018, showed that dietary trehalose at doses above 10 mM enhances the virulence of epidemic *Clostridium difficile*, making it urgent to reduce trehalose intake [7].

Nanocarriers containing trehalose, where trehalose is integrated either chemically or physically, are currently being explored as an alternative option to free trehalose to enhance its efficacy for the treatment of impaired autophagy-associated disorders (*e.g.*, neurodegenerative diseases, nonalcoholic fatty liver disease (NAFLD) and type 2 diabetes, cancer, and atherosclerosis),

which has been reviewed in [**P1**] [6]. The field is relatively new, with reports mostly published after 2017, leaving many unknowns, limitations, and challenges to be addressed.

A total of 8 studies on trehalose-bearing carriers to stimulate autophagy have been published recently, which include nanocarriers with physically entrapped trehalose (*e.g.*, mesoporous silica nanoparticles and β-cyclodextrin-based nanoassemblies) [8], [9] and nanocarriers with chemically bound trehalose (*e.g.*, nanoassemblies [10], [11], [12], [13] and solid-lipid nanoparticles [14] and glycopolymers [15]). The chemical conjugation of trehalose can be either labile or permanent. The first approach relies on covalent bond which can be cleaved under biologically relevant conditions triggering trehalose release. On the other hand, the second strategy implies trehalose being permanently bound to the carrier, making it non-releasable. In this scenario, "poly-(trehalose)" species, which are composed of several copies of trehalose in a single macromolecule or nanoparticle, are currently the main subject of investigation.

3. Main results

The research on trehalose-releasing nanogels has started from the elaboration of the optimal polymerization conditions for nanogel synthesis by FRP using inverse miniemulsion technique. This stage was focused on the synthesis aspects and concerned nanogels without trehalose. As a result of this elaboration cationic and anionic nanogels were developed, which were then studied as carriers for model therapeutics: anionic nanogels for doxorubicin encapsulation ([**P2**] [16]) and cationic nanogels for miRNA complexation ([**P3**] [17]). Further studies presented in [**P4**] [18] and [**P5**] [19] concerned nanogels containing trehalose, which was introduced to polymer network through self-synthetized trehalose monomers: 6-*O*-(meth)acryloyl-trehalose. The main goals concerning their characteristics were to create nanogels with high content of conjugated trehalose, which can be sustainably released at pH 7.4 and which are characterized by high colloidal stability in serum-enriched cell media, hemocompatibility, lack of cytotoxicity, and good cellular uptake. The influence of the composition of nanogels on their physicochemical characteristics and trehalose release was thoroughly investigated. Finally, having established non-cytotoxic and non-hemolytic property of nanogels, their ability to induce autophagy in *in vitro* and *in vivo* models was evaluated, particularly to induce autophagy in transgenic zebrafish and *Drosophila* larvae ([**P4**] [18]) as well as their potential therapeutic applications to treat atherosclerosis ([**P5**] [19]).

3.1. Elaboration of free radical polymerization in inverse miniemulsion for nanogel synthesis

Nanogels were synthesized *via* photoinitiated free radical polymerization (FRP) in inverse miniemulsion. The polymerization occurs in the miniemulsion droplets to afford polymeric nanoparticles with nanoparticle diameter within the diameter range of the droplets, which are generally about 50–500 nm. The organic phase consisted of cyclohexane and Span 80 as emulsifier, while the aqueous phase was composed of phosphate buffer, monomers (non-ionic and ionic), crosslinker, photoinitiator and NaCl as osmotic pressure agent. The presence of ionic monomer was important to provide further colloidal stability of nanogels through electrostatic repulsion. Key parameters in nanogel synthesis, which were optimized included: light irradiation wavelength, irradiation direction (bottom-up or top-down), irradiation time, initiator concentration, stirring condition, aqueous to organic phase (w/o) ratio, and monomer concentration in aqueous phase. The selected polymerization conditions included: bottom-up and 30 min of 395–405 nm light irradiation, 1% w/w LAP initiator, polymerization without stirring, 1:10 v/v of w/o emulsion, and 20% w/v monomer concentration in aqueous phase. The optimization stage resulted in the preparation of anionic and cationic nanogels, which were then studied as nanocarriers for model ionic therapeutics: DOX and miRNA, respectively.

3.2. Trehalose-containing nanogels

The effort to covalently attach trehalose to polymers in a way that allows its controlled release at pH 7.4 is quite challenging due to the limited functional groups available, which are only hydroxyl groups. One way to involve hydroxyl group for conjugation is through an ester linkage. The utility of the ester bond to form labile conjugation is limited because hydrolysis rates of esters at physiologically relevant conditions are generally very slow [20]. However specific structural features can boost the hydrolysis rate. Very recently, our group have found that in polymeric networks of hydrogels fabricated from acrylamides and acrylates, primary or secondary acrylamide-type units significantly accelerate hydrolysis of ester moiety in acrylate units [21]. Utilizing trehalose mono-/diacrylate, this effect was further exploited to develop bulk trehalose-rich hydrogels capable of sustained trehalose release at pH 7.4 [22]. On this basis, herein we have moved to nanoscale and aimed to develop trehalose-releasing nanogels, which could potentially serve as nanocarriers to deliver trehalose as a potent autophagy inducer.

3.2.1. Synthesis and physicochemical characterization of nanogels

Nanogels were synthetized by applying the optimal conditions established previously and described in chapter 3.1 The set of monomers for synthesis of nanogels included four type of monomers: (A) trehalose-incorporating monomer: TreA or TreMA (self-synthetized), (B) non-ionic monomer: acrylamide (AM) - primary amide or DMAM – tertiary amide, (C) ionic monomer: 3-acrylamidopropyltrimethylammonium chloride (AMPTMAC) – secondary acrylamide with quaternary ammonium moiety or ATC – acrylate with quaternary ammonium moiety or 4-acrylamidobutanoic acid (4-AMBA) - secondary acrylamide with carboxylic acid group, and (D) crosslinker: MBA – bis-(secondary acrylamide) or poly(ethylene glycol)$_{400}$ diacrylate (PEG$_{400}$DA) – bis-acrylate (**Figure 1A–D**).

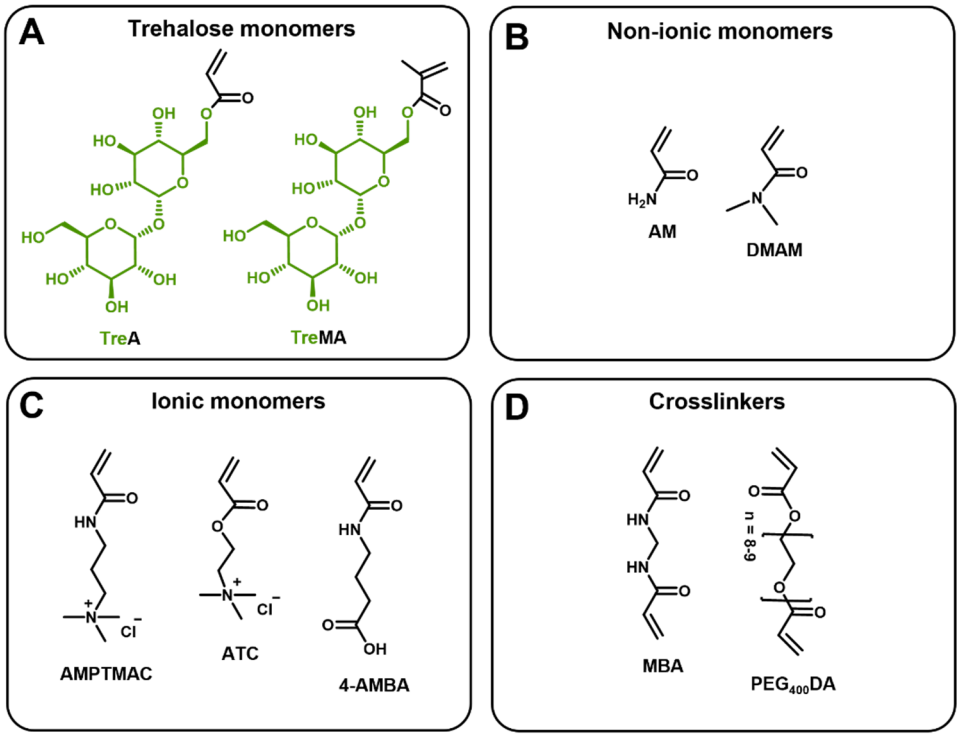

Figure 1. Monomers used for the synthesis of trehalose-containing nanogels. (A) Trehalose monomers: TreA and TreMA. (B) Non-ionic monomers: AM and DMAM. (C) Ionic monomers: AMPTMAC, ATC, and 4-AMBA. (D) Crosslinkers: MBA and PEG$_{400}$DA.

The selection of monomers is crucial for the characteristic of nanogels in terms of releasable or non-releasable trehalose as well as network charge. Combining trehalose (meth)acrylate with secondary and primary acrylamides (AM and AMPTMAC or AMBA and MBA) should enable trehalose release at pH 7.4. In turn replacing these acrylamides with tertiary acrylamide (DMAM) and acrylates (ATC and PEG400DA), which do not accelerate ester hydrolysis, should not induce trehalose release at pH 7.4. Ionic monomers functionalize nanogels with positive (AMPTMAC or ATC) or negative (AMBA) network charge, and one of the reasons

for their introduction was to provide colloidal stability of nanogels. Network charge also offers further possibility of electrostatic binding with bioactive compounds, what could extend nanogels functionality.

Several nanogels described in [**P4**] [18] were synthetized by varying TreA, AMPTMAC, and AM content to study the effect of nanogels composition on trehalose release profile, cytotoxicity, colloidal stability, and to select the optimal composition of trehalose-releasing cationic nanogels.

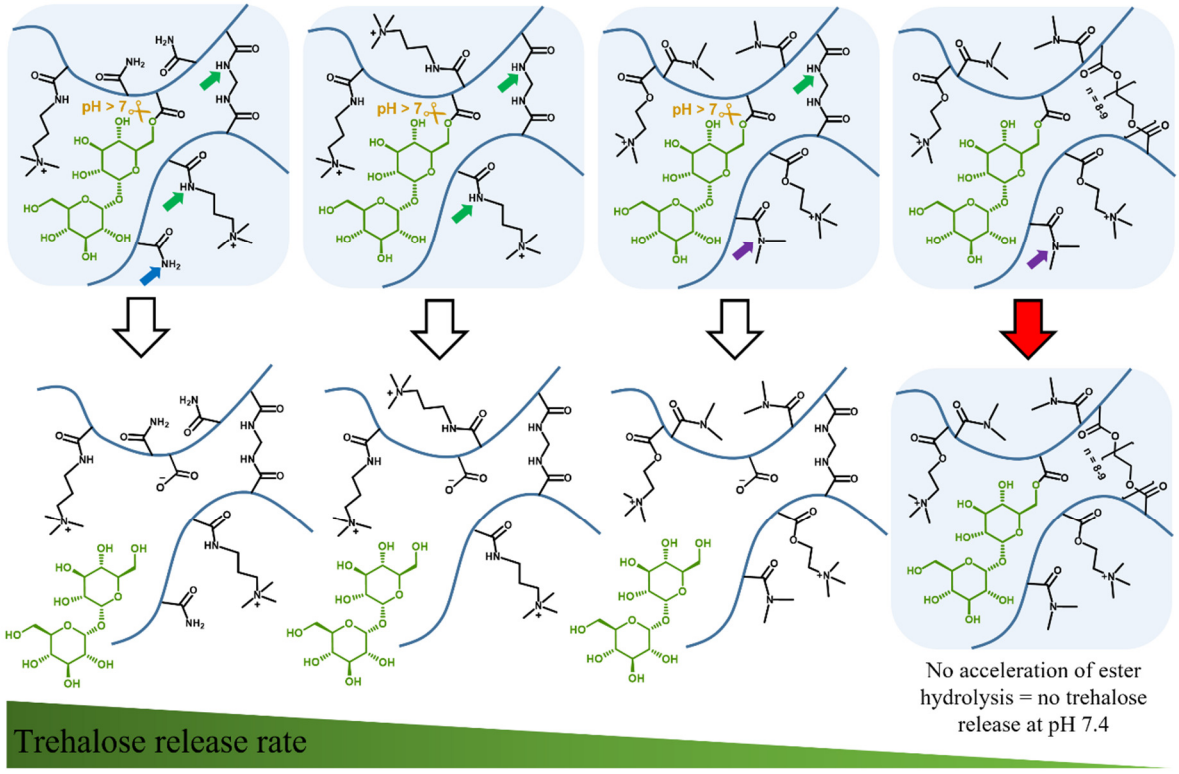

Figure 2. The impact of primary (blue arrow), secondary (green arrow), and tertiary (purple arrow) acrylamide-type co-monomers on ester hydrolysis-mediated trehalose release at pH 7.4.

The replacement of primary and secondary acrylamide-type monomers by acrylates and tertiary acrylamide was done to remove an accelerating effect on ester hydrolysis in acrylate units, and hence obtain nanogels with covalently bound but non-releasable trehalose, which could serve as biological controls for trehalose-releasing nanogels. The illustration on how these acrylamide-based neighboring groups influenced the release of trehalose in physiological conditions is shown in **Figure 2**.

Covalent incorporation of trehalose into nanogels and their purity was confirmed by ^{1}H NMR spectroscopy. The exact content of trehalose in nanogels was determined enzymatically, following its complete liberation into solution by strong alkali treatment. The content of

trehalose was in range between 27.6 and 58.6% w/w. Generally, the amount of incorporated trehalose correlated well with trehalose monomer feed.

The size of trehalose nanogels was determined *via* DLS in DMEM selected as commonly utilized biological medium. Hydrodynamic diameter varied from 50 to 265 nm (PdI range: 0.18–0.3). Cryo-TEM observation revealed that trehalose-releasing nanogels had spherical shape, with average diameter of about 50 nm, which is smaller than the d_H determined by DLS. Nanogels demonstrated varied ζ-potential reaching -5.7 mV for nanogel containing no ionic units, -17.6 mV for nanogel with anionic carboxylic acid moieties and ranging from +22.6 to +41.5 mV for cationic nanogels containing quaternary ammonium cations in the AMPTMAC units.

The colloidal stability of nanogels in serum is paramount important, especially for conducting *in vitro* and *in vivo* studies. It must be ensured that nanogels do not aggregate, since this might lead to toxicity to the cells and impair their capacity to perform as nanocarriers. Generally, nanogels with trehalose content more than 45% were found to be colloidally stable in serum-containing media. Interestingly, trehalose greatly improved the colloidal stability of nanogels, what has been demonstrated by replacing trehalose monomer with 2-hydroxyethyl acrylate (HEA) monomer (hydrophilic monomer with one hydroxyl group), as described in publication [**P5**] [19]The improved colloidal stability of nanogels with high content of trehalose might be influenced by its great hydrophilicity and a large number of hydroxyl groups, reflecting a stabilization mechanism based on short-range repulsive hydration forces [23]. The colloidal stability of the cationic trehalose-releasing nanogels in serum has been found to significantly influence the cytotoxicity profile of the nanogels. Colloidally unstable nanogels were significantly more cytotoxic compared to the stable ones as tested in primary human umbilical vein endothelial cells (HUVECs) up to relatively a high concentration of 1.0 mg/mL [**P4**] [18].

3.2.2. Trehalose release study

The content and the structure of the acrylamide-type co-monomers significantly affected the hydrolysis rate of ester moieties in acrylate units and thus trehalose release (**Figure 3A–C**). Acceleration of ester hydrolysis in acrylate units by acrylamide-type units depends on amide order, and is more prominent for primary amide than for secondary amide, thus the release rate was greatly accelerated when AM was present in nanogel network.

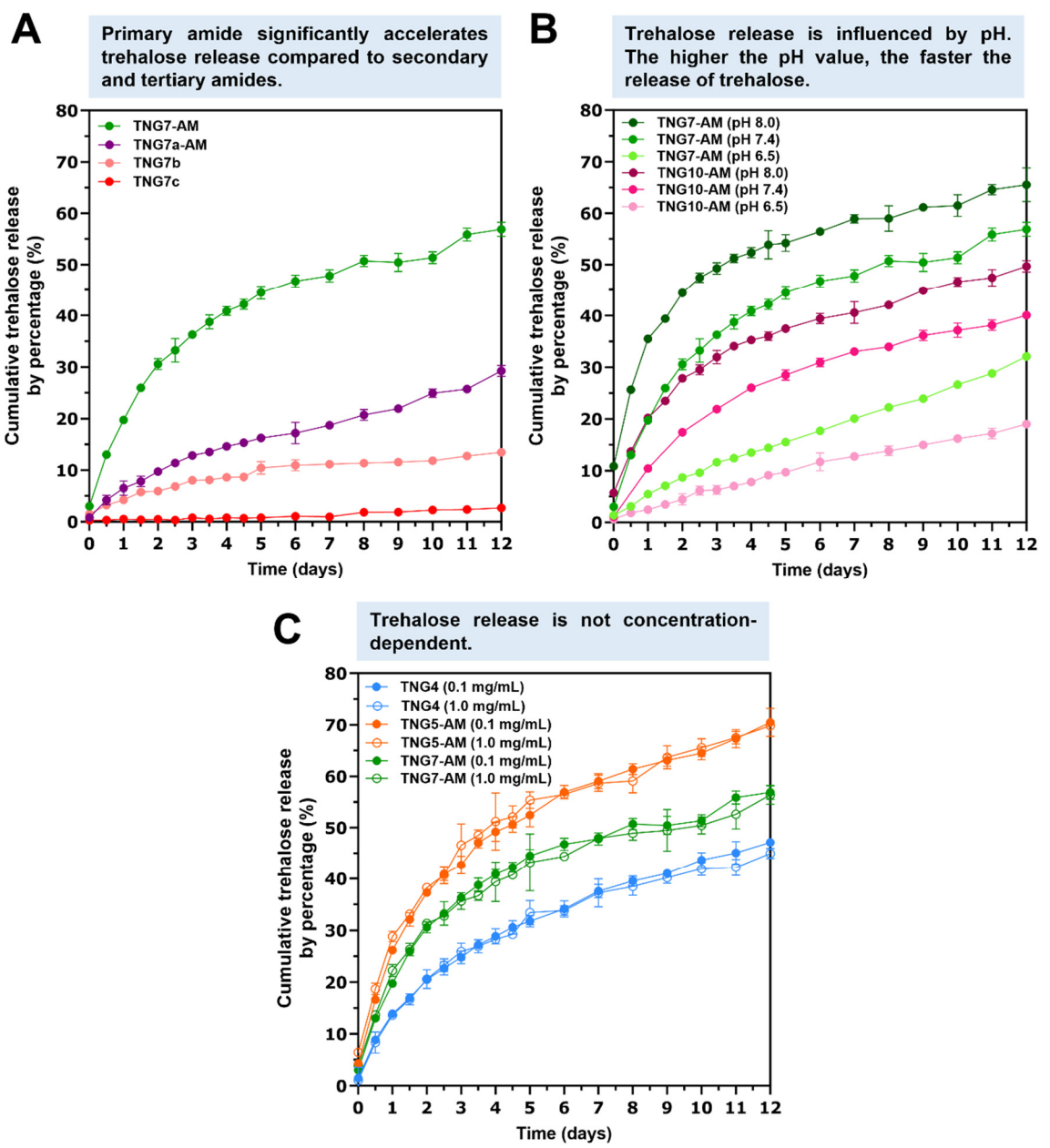

Figure 3. Trehalose release profiles of trehalose-containing nanogels for 12 days in PBS (pH 7.4) at 37 °C. (A) Trehalose release profile from cationic TNG7-AM and its counterparts (TNG7a-AM, TNG7b, and TNG7c). (B) Trehalose release profile from cationic TNG7-AM and anionic TNG10-AM at different pH (6.5, 7.4, and 8.0). (C) Trehalose release profile from TNG4, TNG5-AM, and TNG7-AM at different concentrations (0.1 and 1.0 mg/mL).

In **Figure 3A**, trehalose release profile for TNG7-AM was compared with that of its counterparts TNG7a-AM, TNG7b and TNG7c. The percentage of trehalose release was approximately two to three times lower for nanogel TNG7a-AM with trehalose methacrylate units compared to nanogel TNG7-AM with trehalose acrylate units. The observed differences are consistent with the existing literature indicating that methacrylate-based polymers are by far more resistant to alkaline hydrolysis than acrylate-based polymers [24], [25]. Meanwhile, TNG7b exhibited marginal trehalose release and TNG7c almost no release of trehalose. The modification of the composition of nanogels and replacement of AM and AMPTMAC by co-

monomers which do not accelerate ester hydrolysis in acrylate units (tertiary acrylamide DMAM and acrylate type monomer ATC, respectively) leads to the reduction of trehalose release. To completely avoid trehalose release it is important to replace also MBA crosslinker, as the presence of secondary acrylamide moieties in MBA crosslinker in TNG7b was still enough to provide some trehalose release. The almost zero percent of trehalose release was achieved when $PEG_{400}DA$ was used for crosslinking TNG7c instead of MBA, thus creating trehalose non-releasing nanogel, which could serve as a control in biological environment.

Two oppositely charged nanogels, TNG7-AM and TNG10-AM, were included to investigate how network charge of nanogels and pH of the environment influence the release of trehalose from nanogels. The results of trehalose release profiles from both nanogels provide a clear pH-dependent release rate, where the higher the pH, the faster the release rate (**Figure 3B**). The release from the cationic nanogel TNG7-AM was significantly faster than that from the anionic counterpart TNG10-AM, which might be due to the differences in local pH within nanogel network. Faster rate of ester hydrolysis in hydrogel network containing positively charged moieties compared to the one with negatively charged groups has also been previously observed in literature [26].

The last study on trehalose release assessed comparison of trehalose release from TNG4, TNG5-AM, and TNG7-AM at different concentrations. The results showed that the amount of released trehalose was proportional to nanogel concentration but the release rate was not concentration-dependent (0.1 *vs* 1.0 mg/mL, **Figure 3C**). This property is highly desirable for determining the optimal dose for any *in vitro* or *in vivo* studies.

The successful and sustained release of trehalose from the nanogels at pH 7.4, 37 °C is also well evidenced in ^{1}H NMR spectra, as shown on the example of cationic nanogel (**Figure 4A−C**). With increasing incubation time, the intensity of sharp peaks corresponding to protons of free (released) trehalose was increasing, whereas that of the broad peaks originating from protons of trehalose bound to the nanogel network was decreasing.

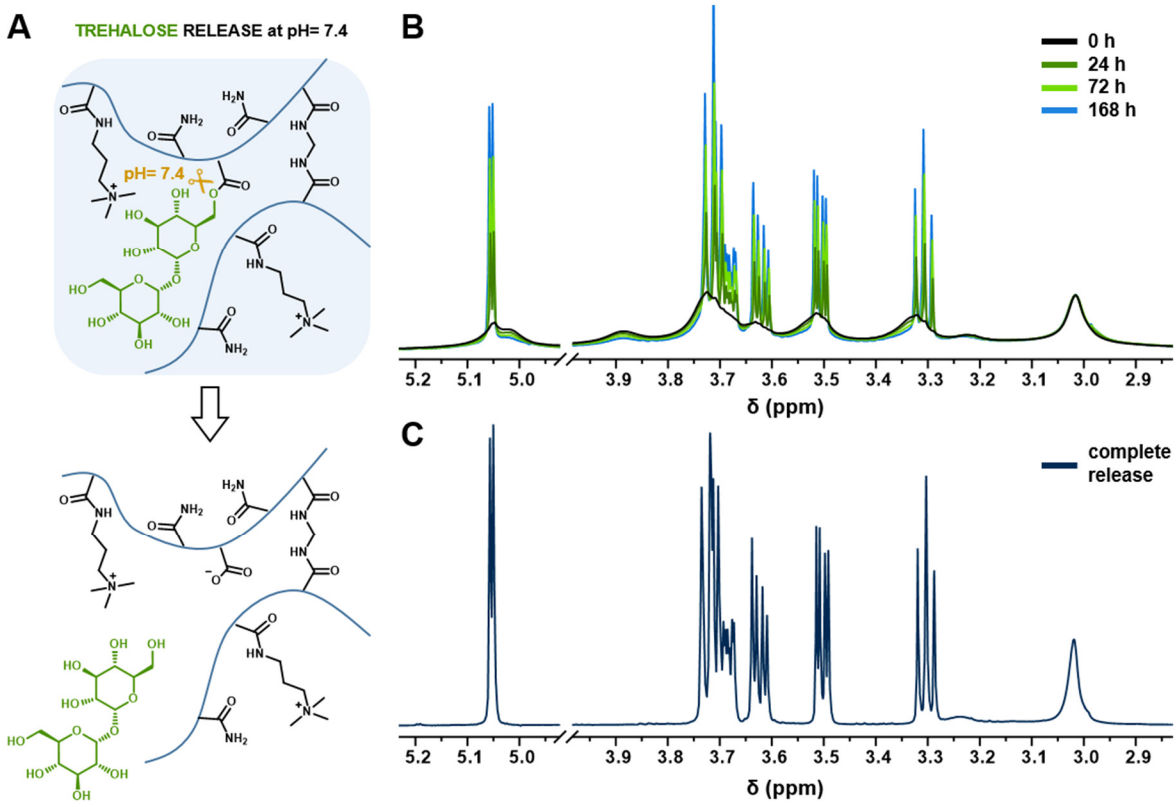

Figure 4. Trehalose release from TNG7-AM (10 mg/mL) evidenced by ^{1}H NMR spectroscopy. (A) Schematic presentation of trehalose release from the nanogel network *via* the hydrolytic cleavage of the ester bond at pH 7.4. (B) Section of ^{1}H NMR spectra of TNG7-AM before and during the release of trehalose at pH 7.4, 37 °C. All spectra are normalized to the signal at 3.0 ppm corresponding to $-CH_3$ protons from AMPTMAC units. (C) Section of ^{1}H NMR spectrum of TNG7-AM after complete release of trehalose induced by the treatment of TNG7-AM with 1M NaOH at 70°C for 1 h. Reproduced with permission from ref. [18]. Copyright 2022 Elsevier.

3.3. Biological studies on trehalose-releasing nanogels

3.3.1. Autophagy stimulation in *Drosophila* and zebrafish larvae

Two trehalose-releasing nanogels: cationic TNG7-AM and neutral TNG9-AM were selected for the *in vivo* autophagy stimulation investigation owing to their outstanding characteristics, which was summarized in the spider graphs [**P4**] [18], particularly due to the highest amount of CTre, good colloidal stability in serum, and a reasonable rate of trehalose release. Having confirmed their non-cytotoxic and non-hemolytic property, as well as good cellular uptake, a well-established zebrafish transgenic reporter line was used to observe the impact of prolonged exposure of those nanogels on the mortality of transgenic zebrafish larvae and their ability to induce autophagy. These studies were conducted by Dr. Máté Varga's group from the Eötvös Loránd University (ELTE), Hungary. The results showed that no significant differences in the mortality of transgenic zebrafish larvae were observed during 4-day treatment (between 1 and 5 days post fertilization – dpf) with 500 µg/mL of TNG7-AM or TNG9-AM. When exposed to

TNG7-AM, the treated larvae demonstrated a considerable increase in autophagic activity as evidenced from the green fluorescent protein (GFP)-microtubule-associated protein 1A/1B-light chain 3 (LC3) signal, but no significant increase in GFP-LC3 signal was observed for TNG9-AM treatment (**Figure 5A**, **B**). Furthermore, an elevated degree of Sqstm1/p62 degradation was spotted in larvae treated with TNG7-AM, reflecting an increase in autophagic activity (**Figure 5C**). These findings imply that prolonged exposure to TNG7-AM can enhance autophagic activity in *in vivo* systems without any observable toxicity.

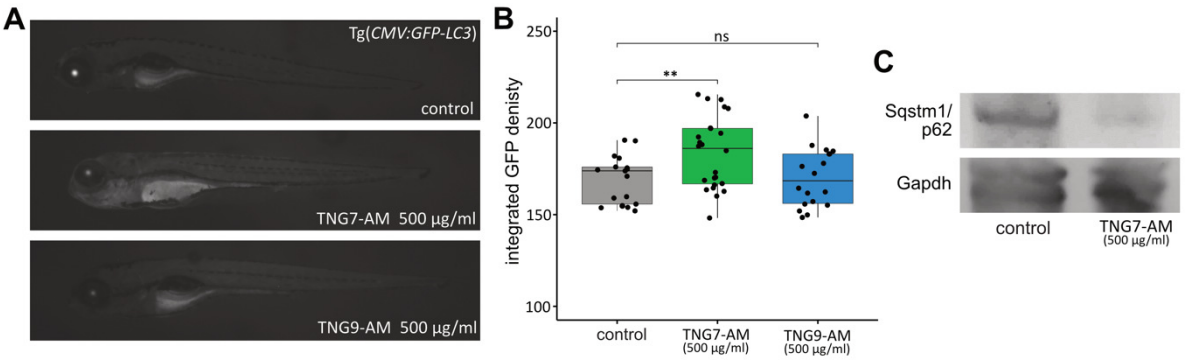

Figure 5. *In vivo* autophagy-enhancing effects of trehalose-releasing nanogels. (A, B) Transgenic zebrafish larvae of the autophagy-reporter line Tg(*CMV:GFP-Lc3*) showed significantly enhanced fluorescence after 4 days of treatment with TNG7-AM, but not with TNG9-AM. (C) Zebrafish treated with TNG7-AM also showed a decrease in Sqstm1/p62 accumulation. Reproduced with permission from ref. [18]. Copyright 2022 Elsevier.

3.3.2. Autophagy stimulation for atherosclerosis treatment in mice

Anionic nanogel called as "TNG" in [**P5**] [19] (**Figure 6A**) was selected to study the potential of trehalose-releasing nanogels to stimulate autophagy in atherosclerosis treatment. This was evaluated both in *in vitro* and *in vivo* studies and was carried out in Prof. Wei Wu's group from Chongqing University, China. To assess nanogel efficacy for inducing autophagy and reducing plaque progression *in vivo*, nanogel and free trehalose were administered intravenously (at doses of 16 mg/kg and 2.5 g/kg, respectively) every three days to ApoE$^{-/-}$ mice with additional high-fat diet (HFD) for 30 days (**Figure 6B**).

After one month of treatment, nanogel demonstrated a significant anti-atherosclerosis effect, reducing the overall plaque area by approximately 60%, surpassing the efficacy of free trehalose. This study is the first report on using nanogels as trehalose carriers to treat atherosclerosis.

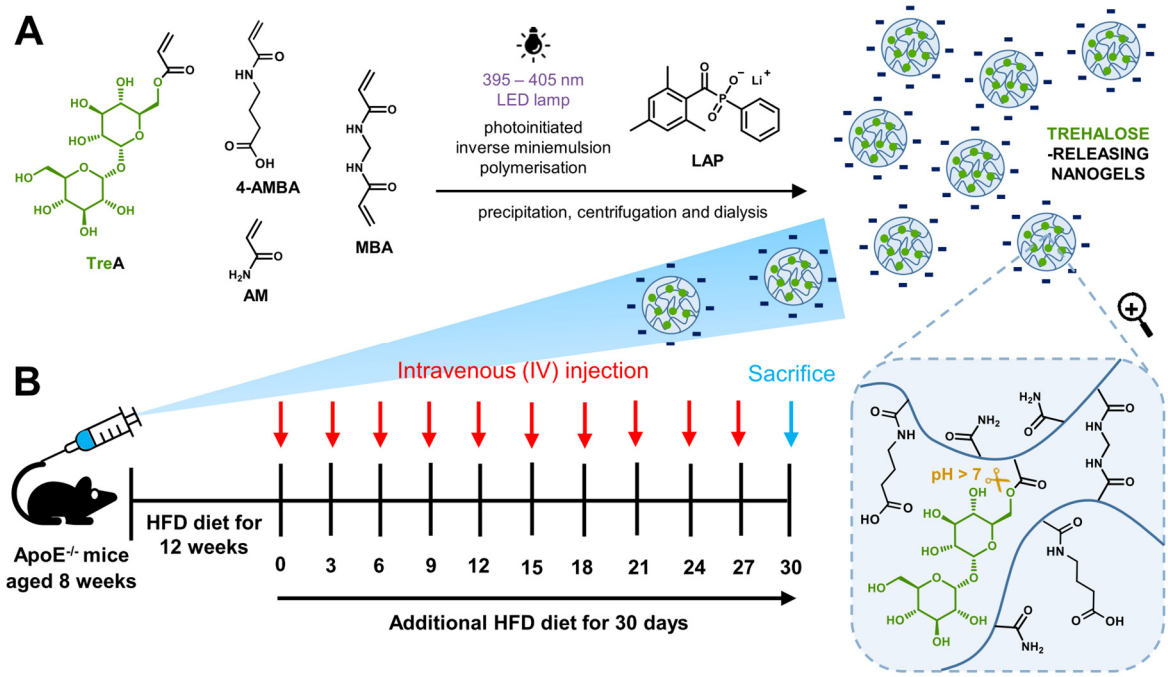

Figure 6. (A) The synthesis of anionic trehalose-releasing nanogels, and (B) timeline for ApoE$^{-/-}$ mice pretreatment and systemic administration of nanogel *via* the tail vein. Reproduced with permission from ref. [19]. Copyright 2023 Springer Nature.

3.4. Fluorescent labeling of nanogels for imaging in biological studies

Fluorescent dyes were incorporated into nanogels by the chemical conjugation using two different approaches (**Figure 7A**, **B**). In the first approach fluorescein *O*-acrylate was incorporated during polymerization, yielding fluorescein-labelled nanogels in one step (**Figure 7A**). In the second approach, fluorescent labeling was carried out in post-polymerization route using reactive moieties installed in nanogels. Specifically self-made 4-AMBA-sulfo-NHS monomer was employed during polymerization to create sulfo-NHS-activated nanogels. These nanogels were then reacted with sulfo-Cy5-amine to form an amide bond and yield Cy5-labelled nanogels (**Figure 7B**).

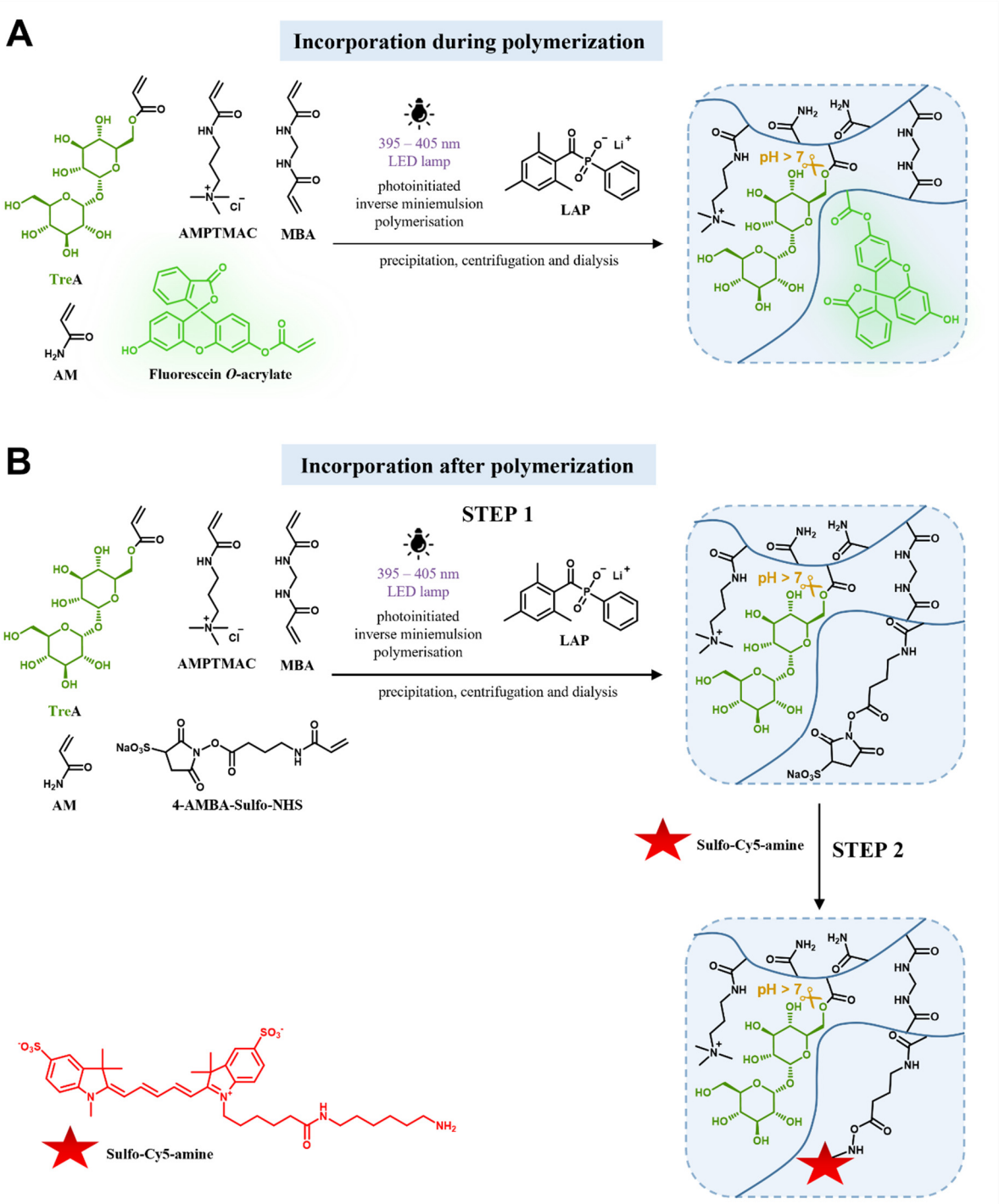

Figure 7. (A, B) The synthesis of fluorescently-labelled trehalose-releasing nanogels using (A) fluorescein *O*-acrylate and (B) sulfo-Cy5-amine as green and red fluorescent agents, respectively.

4. Summary and conclusions

Trehalose is currently used in many biomedical applications, and has a promising potential as autophagy inducer. The construction of covalent, yet hydrolytically-labile at pH 7.4 conjugation of trehalose into nanogels developed as a part of the presented doctoral dissertation is expected to improve its bioavailability and efficacy. The use of FRP in inverse miniemulsion was proved to be suitable method to fabricate uniformly spherical nanogels with appropriate size and plausible yields. Trehalose was covalently conjugated within the polymer network *via* an ester bond, of which the specific location enabled its cleavage under physiologically-relevant conditions resulting in trehalose release. Trehalose release profiles were shown to be dependent on the content and the structure of acrylamide-type units. The lower the percentage of acrylamide-type monomer units in the polymeric network, the slower the release of trehalose from nanogels. The accelerating effect on trehalose release mediated by ester hydrolysis was observed to be more prominent for primary amides than for secondary amides, while it was not observed for tertiary amides. The structure of the acyl moiety, through which trehalose is incorporated within nanogel network, also influenced trehalose release from nanogels. It was found that trehalose release was faster from nanogels containing trehalose acrylate units in comparison to nanogel with trehalose methacrylate units. The network charge of nanogels also influenced trehalose release rate. Cationic nanogels exhibited faster trehalose release than its anionic counterpart, possibly due to the difference in the local pH within the nanogel network. Additionally, trehalose release was depended on pH, with higher pH levels leading to faster release rate. Finally, trehalose release rate does not appear to be concentration-dependent according to the results from three selected nanogels. This property is highly desirable for determining the optimal dose for any *in vitro* or *in vivo* studies. Trehalose presence in nanogels greatly improved their colloidal stability.

As a result of the study, thirteen trehalose-containing nanogels have been successfully synthesized, of which twelve can be classified as trehalose-releasing nanogels and one as trehalose non-releasing nanogel. They were characterized by d_H ranging from 57 to 266 nm and positive or negative zeta potential depending on the charge of the incorporated ionic moieties. The selected trehalose-releasing nanogels had high trehalose content (~50% w/w CTre), were colloidally stable in serum-enriched cell media, non-cytotoxic to HUVECs, and non-hemolytic to human RBCs. More importantly, trehalose-releasing nanogels could significantly induce autophagy in transgenic zebrafish and *Drosophila* larvae and they demonstrated the therapeutic

effects of autophagy stimulation in promoting lipid efflux and plaque reduction in a mouse model of atherosclerosis.

The study was of a high degree of novelty as such trehalose-releasing nanogels had not yet been created and tested its autophagy stimulation effects before. Moreover, it represents a significant achievement in the field of trehalose-bearing carriers, because nanocarriers characterized by covalent, yet labile conjugation of trehalose with its proved sustained release at pH 7.4 have not been developed so far.

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plaque for treatment of atherosclerosis," *ACS Nano* 2022, DOI: 10.1021/acsnano.1c08391.

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6. Academic achievements related to doctoral dissertation

Publications:

1. [**P1**] Maruf A*, Milewska M*, Varga M, Wandzik I*. *Trehalose-Bearing Carriers to Target Impaired Autophagy and Protein Aggregation Diseases*. **J. Med. Chem.** 2023, 66, 15613−15628. DOI: 10.1021/acs.jmedchem.3c01442.
   (IF_{2022} = 7.300, MEiN = 200 point, *contributed equally)
2. [**P2**] Maruf A, Milewska M, Lalik A, Wandzik I. *pH and Reduction Dual-Responsive Nanogels as Smart Nanocarriers to Resist Doxorubicin Aggregation*. **Molecules**. 2022, 27, 5983. DOI: 10.3390/molecules27185983.
   (IF_{2021} = 4.927, MEiN = 140 point)
3. [**P3**] Maruf A, Milewska M, Lalik A, Student S, Wandzik I. *A Simple Synthesis of Reduction-Responsive Acrylamide-Type Nanogels for miRNA Delivery*. **Molecules**. 2023, 28, 761. DOI: 10.3390/molecules28020761.
   (IF_{2022} = 4.600, MEiN = 140 point)
4. [**P4**] Maruf A, Milewska M, Kovács T, Varga M, Vellai T, Lalik A, Student S, Borges O, Wandzik I. *Trehalose-releasing nanogels: A step toward a trehalose delivery vehicle for autophagy stimulation*. **Biomater. Adv.** 2022, 138, 212969. DOI: 10.1016/j.bioadv.2022.212969.
   (formerly known as **Mater. Sci. Eng. C** with IF_{2021} = 7.328, MEiN = 140 point)
5. [**P5**] Zhong Y*, Maruf A*, Qu K, Milewska M, Wandzik I, Mou N, Cao Y, Wu W. *Nanogels with covalently bound and releasable trehalose for autophagy stimulation in atherosclerosis*. **J. Nanobiotechnol.** 2023, 21, 472. DOI: 10.1186/s12951-023-02248-9.
   (IF_{2022} = 10.200, MEiN = 140 point, *contributed equally)

Conferences:

1. Maruf A, Milewska M, Salvati A, Wandzik I. The surface charge effects of trehalose-releasing nanogels on trehalose release, protein corona formation, and cell uptake. Oral presentation at Pharmacy Day 2024, The University of Groningen. 11 June 2024, Groningen, The Netherlands.
2. Maruf A, Milewska M, Wandzik I. Synthesis and characterization of trehalose-containing nanocarriers for potential cancer treatments. Poster presentation at the 27th Gliwice Scientific Meetings. 16-17 November 2023, Gliwice, Poland.

3. Maruf A, Lalik A, Milewska M, Wandzik I. Nanoparticles as intracellular trehalose delivery systems for cryopreservation. Poster presentation at the 12th International Colloids Conference (Elsevier). 11-14 June 2023, Palma, Spain.
4. Maruf A, Lalik A, Milewska M, Wandzik I. Polymers releasing trehalose as a potential solvent-free cell banking. Oral presentation at Computational Oncology and Personalized Medicine – Crossing Borders, Connecting Science (COPM2023) Conference. 26th April 2023, Gliwice, Poland.
5. Maruf A, Milewska M, Lalik A, Wandzik I. Smart pH/redox dual-responsive nanogels as doxorubicin delivery systems. Poster presentation at the 26th Gliwice Scientific Meetings. 18-19 November 2022, Gliwice, Poland.
6. Maruf A, Milewska M, Lalik A, Wandzik I. Glutathione-responsive nanogels as drug delivery systems. Oral presentation at NanoTech Poland 2022. 1-3 June 2022, Poznan, Poland.
7. Maruf A, Milewska M, Lalik A, Student S, Wandzik I. Trehalose-releasing nanogels: a potential trehalose delivery system for autophagy stimulation. Oral presentation at the 25th Anniversary of Gliwice Scientific Meetings: Joint online seminar of the Polish Proteomics Society and the Finnish Proteomics Society: Proteomics of Extracellular Vesicles. 18-20 November 2021, Gliwice, Poland.
8. Milewska M, Maruf A, Waśkiewicz S, Lalik A, Student S, Wandzik I. Micro- and nanosized hydrogels for biomedical applications. Co-author of the short lecture by Prof. Ilona Wandzik at the 25th Anniversary of Gliwice Scientific Meetings: Joint online seminar of the Polish Proteomics Society and the Finnish Proteomics Society: Proteomics of Extracellular Vesicles. 18-20 November 2021, Gliwice, Poland.
9. Maruf A, Milewska M, Lalik A, Student S, Wandzik I. A Series of Trehalose-Releasing Polymers as Potential Drug Delivery Agents for Autophagy Stimulation. Poster presentation at the 7th Young Polymer Scientists Conference and Short Course. 27-28 September 2021, Lodz, Poland.

Project:

Title of project: Trehalose releasing nanogels for autophagy stimulation (PRELUDIUM BIS 1, Grant No. 2019/35/O/ST5/02746, The National Science Center (NCN), Poland)

Project duration: 01/10/2020 to 30/09/2024 (48 months)

The function performed in the project: main contractor (project manager: prof. dr hab. inż. Ilona Wandzik)

Patent application:

Ilona Wandzik, Małgorzata Milewska, Ali Maruf; Sposób otrzymywania nanożeli uwalniających kowalencyjnie związaną trehalozę. Patent application number: P.438753, submission date: 16 August 2021, Urząd Patentowy RP, Warszawa, Poland.

Awards:

Pro-quality scholarship for the best PhD students of The Silesian University of Technology in four academic years (2020/2021, 2021/2022, 2022/2023, and 2023/2024), under the implementation of The Excellence Initiative – Research University (IDUB, 2020–2026) program (08/IDUB/2019/94).

Foreign internships:

1. Research Internship at The University of Helsinki, Finland; Duration: 6 months, from 01.02.2023 to 01.08.2023; Foreign supervisor: Prof. Mikko Airavaara; Research activities: conducting research on trehalose-releasing nanogel effects on *in vivo* model of Parkinson's Diseases as part of Project PRELUDIUM BIS 1 (2019/35/O/ST5/02746) from NCN, Poland; The mobility was funded by NAWA (PPN/STA/2021/1/00053), external grant.
2. Research Internship at The University of Coimbra, Portugal; Duration: 2 weeks, from 05.09.2021 to 15.09.2021; Foreign supervisor: Dr. Olga Borges; Research activities: conducting research on investigation of the hemolysis property of trehalose-releasing nanogels on human red blood cells as part of Project PRELUDIUM BIS 1 (2019/35/O/ST5/02746) from NCN, Poland; The mobility was funded by NAWA under PROM program (POWR.03.03.00-IP.08-00-P13/18), managed by The Silesian University of Technology.
3. Research Internship at The University of Groningen, the Netherlands; Duration: 3 months, from 02.04.2024 to 28.06.2024; Foreign supervisor: Prof. Anna Salvati; Research activities: conducting research on investigation of the effects of cationic, anionic, and zwitterionic trehalose-releasing nanogels on protein corona formation and uptake mechanisms as part of Project PRELUDIUM BIS 1 (2019/35/O/ST5/02746) from NCN, Poland; The mobility was funded by The Silesian University of Technology through the mobility scholarships for the best PhD students of The Silesian University of Technology (2023/2024 academic year, Decision no. RJO15.5033.455.2023) under the implementation of The Excellence Initiative – Research University (IDUB, 2020–2026) program (08/IDUB/2019/94) co-financed with the PRELUDIUM BIS 1 project (2019/35/O/ST5/02746).