Journal of Visualized Experiments

A non-degradative extraction method for fine molecular structure characterization of bacterial glycogen particles

--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE63016R4
Full Title:	A non-degradative extraction method for fine molecular structure characterization of bacterial glycogen particles
Corresponding Author:	Liang Wang Xuzhou Medical University Xuzhou, Jiangsu Province CHINA
Corresponding Author's Institution:	Xuzhou Medical University
Corresponding Author E-Mail:	healthscience@foxmail.com
Order of Authors:	Jun-Jiao Wang
	Meng-Meng Wang
	You-Wei He
	Zhang-Wen Ma
	Zi-Yi Wang
	Rui Qiao
	Xin-Le Tan
	Qing-Hua Liu
	Liang Wang
Additional Information:	
Question	**Response**
Please specify the section of the submitted manuscript.	Biology
Please indicate whether this article will be Standard Access or Open Access.	Standard Access ($1400)
Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below:	I agree to the Author License Agreement
Please confirm that you have read and agree to the terms and conditions of the video release that applies below:	I agree to the Video Release
Please provide any comments to the journal here.	
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	APF

TITLE:

A Non-Degradative Extraction Method for Molecular Structure Characterization of Bacterial Glycogen Particles

AUTHORS AND AFFILIATIONS:

Jun-Jiao Wang[1,#], Meng-Meng Wang[2,3,#], You-Wei He[4], Zhang-Wen Ma[2,3], Zi-Yi Wang[5,6,7], Rui Qiao[2,3], Xin-Le Tan[5,6], Qing-Hua Liu[8]*, Liang Wang[1,2]*

[1]Department of Bioinformatics, School of Medical Informatics and Engineering, Xuzhou Medical University, Xuzhou, Jiangsu Province, 221000, China.
[2]Jiangsu Key Laboratory of New Drug Research and Clinical Pharmacy, School of Pharmacy, Xuzhou Medical University, Xuzhou, Jiangsu Province, 221000, China.
[3]Department of Pharmaceutical Analysis, School of Pharmacy, Xuzhou Medical University, Xuzhou, Jiangsu Province, 221000, China.
[4]Department of Biological Sciences, School of Life Sciences, Xuzhou Medical University, Xuzhou, Jiangsu, 221000, China.
[5]School of Chemistry and Molecular Biosciences, The University of Queensland, Brisbane, Queensland, 4072, Australia.
[6]Centre for Nutrition and Food Sciences, Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, Brisbane, Queensland, 4072, Australia.
[7]Joint International Research Laboratory of Agriculture and Agri-Product Safety, College of Agriculture, Yangzhou University, Yangzhou, Jiangsu Province, 225009, China.
[8]State Key Laboratory of Quality Research in Chinese Medicines, Macau University of Science and Technology, Avenida Wai Long, Taipa, Macau 999078, China.

[#]These authors contributed equally to the study.

*Corresponding Authors:
Qing-Hua Liu (qhliu@must.edu.mo)
Liang Wang (healthscience@foxmail.com)

Email addresses of co-authors:
Jun-Jiao Wang (1454939318@qq.com)
Meng-Meng Wang (642278929@qq.com)
You-Wei He (1805043643@qq.com)
Zhang-Wen Ma (mzw19980402@163.com)
Zi-Yi Wang (ziyi.wang2@uqconnect.edu.au)
Rui Qiao (1395907520@qq.com)
Xin-Le Tan (xinle.tan@uq.edu.au)
Qing-Hua Liu (qhliu@must.edu.mo)
Liang Wang (healthscience@foxmail.com)

SUMMARY:

Bacterial glycogen structure is greatly impacted by extraction methods which may result in

molecular degradation and/or biased sampling. It is essential to develop methods to minimize these problems. Here, four extraction methods have been compared using size distribution and chain length distribution as key criteria for minimizing extraction artifacts.

ABSTRACT:

Currently, there exist a variety of glycogen extraction methods, which either damage glycogen spatial structure or only partially extract glycogen, leading to the biased characterization of glycogen fine molecular structure. To understand the dynamic changes of glycogen structures and the versatile functions of glycogen particles in bacteria, it is essential to isolate glycogen with minimal degradation. In this study, a mild glycogen isolation method is demonstrated by using cold-water (CW) precipitation *via* sugar density gradient ultra-centrifugation (SDGU-CW). The traditional trichloroacetic acid (TCA) method and potassium hydroxide (KOH) method were also performed for comparison. A commonly used lab strain, *Escherichia coli* BL21(DE3), was used as a model organism in this study for demonstration purposes. After extracting glycogen particles using different methods, their structures were analyzed and compared through size exclusion chromatography (SEC) for particle size distribution and fluorophore-assisted capillary electrophoresis (FACE) for linear chain length distributions. The analysis confirmed that glycogen extracted *via* SDGU-CW had minimal degradation.

INTRODUCTION:

Glycogen is a highly branched polysaccharide that consists of glucosyl residues and also a small but significant amount of proteins, in which all glucosyl residues are linked together *via* α-1,4-glycosidic bonds in linear chains and α-1,6-glycosidic bonds at branching points[1]. The structure of glycogen particles is generally divided into three hierarchies: 1) short-chain oligomers, 2) spherical β particles (~20 nm in diameter), and 3) large rosette-shaped α particles aggregated together by β particles, the diameter of which ranges roughly up to 300 nm. Recently, it has been found that glycogen α particles have two structural states in eukaryotes, i.e., a fragile state and a stable state. Here, fragility means the dissociation of larger α particles into smaller β particles in the presence of a chaotropic agent like DMSO[2]. Further analyses found that glycogen α particles in the diabetic liver are consistently fragile[3] and the fragile α particles degrade much faster than stable α particles[4]. Thus, glycogen structural fragility may exacerbate hyperglycemic conditions in diabetes[2,4], which makes fragile α-particle a potential pathological biomarker of diabetes at a molecular level. However, the existence of glycogen α particles in prokaryotes is only sporadically reported[5], and there is no report of the two different structural states of glycogen α particles in bacteria.

In order to understand the physiological functions of bacterial glycogen particles, it is essential to determine the fine structure of glycogen molecules, which requires glycogen isolation with maximal yield and minimal degradation[1]. So far, various techniques have been developed for glycogen extraction, including but not limited to hot water extraction, trichloroacetic acid (TCA) extraction, and hot alkaline (potassium hydroxide, KOH) extraction[6]. In addition, another method that is commonly used for eukaryotic glycogen isolation, the sugar density gradient ultra-centrifugation (SDGU) method, was also reported for bacterial glycogen isolation in *Selenomonas ruminantium* and *Fibrobacter succinogenes*[7,8]. Although the pros and cons of

these methods have been widely discussed in eukaryotic studies[9,10], there are rarely comparative studies of glycogen fine structures isolated *via* different extraction methods in bacteria from the perspective of glycogen particle structures.

In this study, this issue has been addressed by using *Escherichia coli* BL21(DE3) as the model organism. A total of four glycogen extraction methods were compared, namely, TCA-precipitated hot water extraction (TCA-HW), TCA-precipitated cold-water extraction (TCA-CW), hot 30% KOH solution extraction (KOH-HW), and cold-water extraction using sucrose density gradient ultracentrifugation (SDGU-CW). Glycogen particle size distribution was then measured *via* size exclusion chromatography (SEC) while chain-length distribution was detected *via* fluorophore-assisted carbohydrate electrophoresis (FACE), both of which were used for assessing the quality of extraction methods. In addition, the stability and fragility of bacterial glycogen α particles were also compared among the various extraction methods by comparing particle size distribution before and after treating with the commonly used chaotropic agent, dimethyl sulfoxide (DMSO). The detailed procedures for glycogen extraction and structural characterization are presented below. In summary, the SDGU-CW method has the best overall effect in terms of glycogen structural integrity and is, therefore, recommended for bacterial glycogen extraction in future relevant studies.

PROTOCOL

1. Bacteria culture and collection

1.1. Resuscitate *E. coli* BL21(DE3) from bacterial glycerol stock (-80 °C) by inoculating sterile LB agar plate (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, and 15 g/L agar). Put the plate into a standard incubator and cultivate overnight at 37 °C.

1.2. Pick up a single colony and inoculate it into a 10 mL sterile LB liquid medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl). Mix well *via* vortexing and culture overnight at 37 °C with shaking at 220 rpm.

NOTE: Unless otherwise specified, all liquid culture conditions were 37 °C with a 220-rpm shaking rate.

1.3 Transfer 1 mL of the overnight *E. coli* culture into 100 mL of sterile LB liquid medium and culture for 5 h. Transfer 50 mL to 1 L of 1x M9 minimal medium (3 g/L KH_2PO_4, 0.5 g/L NaCl, 6.78 g/L Na_2HPO_4, and 1 g/L NH_4Cl) containing 0.8% D-(+)-glucose. Mix well, and culture for 20 h.

NOTE: 1x M9 minimal medium and glucose were sterilized separately and then mixed when cooling down to room temperature aseptically.

1.4 After culturing for 20 h, centrifuge bacterial solution at 6,000 x *g* for 15 min at 4 °C. Discard the supernatant. Store the cell pellet at -80 °C overnight and then freeze-dry the pellet.

1.5 Seal and store the lyophilized bacterial powder in a refrigerator (-20 °C) for later use.

2. Glycogen extraction

2.1. Trichloroacetic acid precipitated hot water extraction (TCA-HW)

2.1.1. Precisely weigh 500 mg of freeze-dried *E. coli* BL21 (DE3) powder and resuspend it in 20 mL of ice-cold 0.05 M triethanolamine (TEA) buffer. Use an ultrasonic cell crusher (25% energy, 4 °C) to disrupt bacterial cells for 3 min (30 s working cycles and 2 s intervals). Ensure that there are no obvious pellets in the solution.

NOTE: Cell disruption was carried out on the ice. Adjust the pH of TEA buffer to pH 7 by HCl addition and store at room temperature.

2.1.2. Transfer all the bacterial homogenate to two 10.4 mL ultracentrifuge tubes (10 mL/ tube). Fill the tubes with deionized water to the top, and centrifuge at 104,000 x *g* in an ultracentrifuge at 4 °C for 90 min. Discard the supernatant.

2.1.3. Add 2 mL of deionized water to resuspend the pellet in each tube. Transfer suspension to a 50 mL centrifuge tube and add deionized water to a final volume of 20 mL. Heat and boil for 5 min to denature all proteins.

2.1.4. Centrifuge the suspension for 10 min at 18,000 x *g* and retain the supernatant (S1). Treat the precipitate the same way as in step 2.1.3. Pool the new supernatant (S2) with S1.

2.1.5. Add 50% TCA (0.1 volume) to the supernatant (S1+S2) and place the mixture on ice for 10 min to precipitate macromolecules such as DNA, RNA, proteins, etc. Centrifuge the mixture at 18,000 x *g* for 10 min and mix the supernatant with 1.5 volume of absolute ethanol.

NOTE: The concentration of TCA is 50% v/v. For storage, keep it away from light.

2.1.6. Precipitate glycogen on ice for 20 min and centrifuge at 18,000 x *g* for 10 min. Pour out supernatant.

2.1.7. Dissolve the pellet in 5 mL of ddH_2O, and then add 5 mL of ice-cold absolute ethanol. Incubate the solution overnight at 4 °C and centrifuge at 18,000 x *g* for 10 min. Discard the supernatant and keep the pellet.

2.1.8. Repeat the “wash and precipitation” steps as in step 2.1.7 two more times.

2.1.9. Finally, dissolve the precipitated glycogen in 400 µL of ddH_2O in a 2 mL tube, pre-freeze at -80 °C, and freeze-dry to obtain dry glycogen powder.

2.2. Trichloroacetic acid precipitated cold water extraction (TCA-CW)

NOTE: TCA-CW method is the same as the TCA-HW method except that, after discarding the supernatant, treat the pellet without boiling (see step 2.1.3) and resuspend it in 20 mL of ddH_2O containing 1 mg/mL protease inhibitor cocktail.

2.2.1. To prepare a 1 mg/mL protease inhibitor cocktail solution, dissolve 50 mg of protease inhibitor cocktail in 1 mL of deionized water and dilute at a ratio of 50:1 (deionized water: protease inhibitor cocktail solution).

2.3. Cold water extraction using sucrose density gradient ultracentrifugation (SDGU-CW)

2.3.1. Dissolve and homogenize 1 g of freeze-dried *E. coli* powder in 4 mL of glycogen extraction buffer (GEB) with a 1 mg/mL protease inhibitor cocktail.

NOTE: GEB consists of 50 mM Tris, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, and 5 mM sodium pyrophosphate. GEB needs to be adjusted to pH 8 with HCl.

2.3.2. Use an ultrasonic cell crusher (25% energy, 4 °C) to disrupt bacterial cells for 3 min (8 s working cycles and 9 s intervals). Ensure that the entire process is carried out on the ice. After sonification, transfer the bacterial homogenate to a centrifuge tube, fill it with GEB to a final volume of 10 mL, and vortex the tube to mix.

NOTE: Do not homogenize for too long a time as heat generated may degrade glycogen.

2.3.3. Centrifuge at 6,000 x *g* for 10 min at 4 °C. Transfer the supernatant to a 10.4 mL ultracentrifuge tube, fill the tube to the top with GEB, and then centrifuge at 360,000 x *g* for 2 h at 4 °C.

NOTE: Make sure that the tube is filled up and no air bubbles exist.

2.3.4. Discard the supernatant after centrifugation and resuspend the precipitate with 2 mL of deionized water.

2.3.5. In a new ultracentrifuge tube, slowly layer 4 mL of 75% sucrose solution with 4 mL of 37.5% sucrose solution. Then, layer the suspension obtained in step 2.3.4 on top of the sucrose solution, and top up with deionized water (see **Figure 1**).

NOTE: The sucrose concentration is 75% [v/v] and 37.5% [v/v]. Be careful when making the sucrose density gradient and ensure that there is an observable layering between the two sucrose solutions. Also, ensure that there are no air bubbles in the ultracentrifuge tube.

2.3.6. Centrifuge at 360,000 x *g* for 2.5 h at 4 °C and discard the supernatant. Dissolve the pellet in 200 µL of deionized water. Add 800 µL of absolute ethanol for glycogen precipitation.

2.3.7. Precipitate glycogen at -20 °C overnight. Centrifuge at 4,000 x *g* for 10 min at 4 °C and discard the supernatant.

2.3.8. Dissolve the resulting pellet in 400 µL of ddH_2O in a 2 mL tube, pre-freeze at -80 °C, and freeze-dry to obtain dry glycogen powder. Preserve dry glycogen powder at 4 °C for structural analysis.

NOTE: The detailed procedure is illustrated in **Figure 1**.

2.4. Hot 30% potassium hydroxide solution extraction (KOH-HW)

2.4.1. Boil freeze-dried *E. coli* powder (50 mg) in 1 mL of 30% [w/v] KOH for 1 h.

2.4.2. Add 67% [v/v] ethanol containing 15 mM LiCl for precipitation at -20 °C for at least 1 h. Centrifuge the samples at 16,000 x *g* at 4 °C for 20 min.

NOTE: 67% [v/v] ethanol with 15 mM LiCl contains 0.6358 g LiCl, 67 mL of absolute ethanol and 33 mL of deionized water.

2.4.3. Redissolve the pellets in 1 mL of ddH_2O and heat for 10 min at 95 °C with intermittent agitation.

2.4.4. Repeat the ethanol precipitation step three more times as described in step 2.4.2. Redissolve the final pellet in 400 µL of ddH_2O in a 2 mL tube, pre-freeze at -80 °C, and freeze-dry to obtain dry glycogen powder.

3. Glycogen structure determination

3.1. Transmission Electron Microscopy (TEM)

3.1.1. Resuspend glycogen powder in 50 mM Tris-buffered saline (pH 7) with a final concentration of 1 mg/mL.

3.1.2. Make a 10-fold dilution of the suspension and apply the diluted suspension onto the glow-discharge 400-mesh copper grid.

3.1.3. After 2 min, draw the excess sample off the grid using a filter paper and stain the grid with 2–3 drops of 1% uranyl acetate.

3.1.4. Use a transmission electron microscope operating at 75 kV to examine the preparations.

3.2. Size Exclusion Chromatography (SEC)

3.2.1. Preparation of mobile phase: prepare 0.02% (w/w) sodium azide and 50 mM sodium nitrate solutions in deionized water, and filter through a 0.45 µm filter membrane. Use the ultrasonic oscillating water bath to sonicate the solution for more than 15 min to remove air bubbles.

3.2.2. Sample preparation: Use the mobile phase to dissolve glycogen powder so that the final concentration is 1 mg/mL. Incubate the solution at 80 °C overnight in a thermomixer. Centrifuge the dissolved sample at 6,000 x *g* for 10 min at room temperature and transfer the supernatant to a standard SEC vial.

3.2.3. Preparation of DMSO-treated glycogen samples

3.2.3.1. Dissolve 1 mg of glycogen powder in 300 µL of DMSO and incubate it at 80 °C overnight in a thermomixer. Add 4x volume of ethanol to precipitate glycogen and centrifuge the solution at 6,000 x *g*.

3.2.3.2. Wash the pellet twice with ethanol, dissolve the pellet in ddH_2O and lyophilize.

3.2.3.3. Analyze the freeze-dried glycogen powder *via* SEC by preparing samples as described above.

3.2.4. Use SEC system with pre-columns, and 1000 and 10000 columns for the analysis of glycogen particle size distribution. Keep the columns at 80 °C and the flow rate at 0.3 mL/min.

3.3. Fluorophore-Assisted Carbohydrate Electrophoresis (FACE)

3.3.1. Glycogen debranching

NOTE: When using FACE to detect chain length distribution (CLD), all samples to be tested need to undergo the pretreatment of debranching.

3.3.1.1. Add 0.5 mg of glycogen powder, 90 µL of hot water (90 °C), 1.5 µL of NaN_3 solution, 3.5 µL of isoamylase (200 U/mL), and 8 µL of acetic acid-sodium acetate buffer (pH 3.5) to a test tube.

NOTE: Isoamylase is added to specifically cut the branched-chain of the sample to be tested. Isoamylase is a debranching enzyme that can specifically destroy the α-(1-6) bond without destroying the α-(1-4) bond, so it can specifically cleave the glycogen side chain without destroying the structure of the main chain.

3.3.1.2. Incubate the mixture at 37 °C for 3 h in a thermomixer. After making the pH neutral with 8 µL of 0.1 M NaOH solution, incubate the mixture at 80 °C for 1 h in a thermomixer.

3.3.1.3. Add 4x volume of absolute ethanol to the mixture to precipitate glycogen. Then,

centrifuge at 6,000 x *g* for 10 min at room temperature. Wash the pellet twice with ethanol, dissolve the pellet into ddH_2O and lyophilize.

3.3.2. Preparation of fluorescent solution

3.3.2.1. Centrifuge the APTS (8-Aminopyrene-1,3,6-trisulfonic acid trisodium salt) reagent bottle (each bottle contains 5 mg APTS) at 4,000 x *g* for 2 min. Add 50 µL of 15% acetic acid solution to dissolve the powder, mix well, and centrifuge at 4,000 x *g* for 2 min at room temperature to obtain 0.2 M APTS acetic acid solution.

NOTE: Keep the APTS solution in a refrigerator at -20 °C. It must be used completely within two weeks once opened. Otherwise, it will be inactivated. APTS is a commonly used negatively charged dye that binds to the reducing end of the glycogen chain. Since the chains with different degrees of polymerization (DP) all carry only one negative charge, FACE can separate them based on their different mass-to-charge ratios. In this way, signals of different DP values are detected by the fluorescence detector.

3.3.3. Transfer the debranched glycogen to a test tube and add 1.5 µL of APTS solution and 1.5 µL of sodium cyanoborohydride solution. Incubate at 60 °C for 1.5 h in the dark. Add 80 µL of deionized water, centrifuge at 4,000 x *g* for 10 min at room temperature and keep the supernatant.

3.3.4. Introduce the sample into the capillary electrophoresis system by injecting for 3 s at 0.5 psi (3.4 kPa above atmospheric pressure).

NOTE: Fluorescently labeled linear glucans were separated through applied voltage of 30 kV and an approximate current of 14 mA at 25 °C. Peak areas gave relative amounts of glucans with different masses (degree of polymerization (DP) of glucans in adjacent peaks differed by 1 DP). The sample temperature was kept at 18 °C.

REPRESENTATIVE RESULTS:

Size distribution of glycogen particles

A series of studies have shown that glycogen α particles in the diabetic liver are fragile and easily broken apart in the hydrogen bond disruptor DMSO[11–14]. The present study tested how particle size and structural stability changed for bacterial glycogen extracted through four different methods. All glycogen samples from the four methods were treated with water (blue curves) and DMSO (red curves), respectively. The weight distributions, $w(\log R_h)$, are given in **Figure 2**. Water-treated glycogen extracted *via* TCA-HW (**Figure 2A,** blue curves) and TCA-CW (**Figure 2B,** blue curves) is dominated by smaller particles with peaks at $R_h \sim 20$ nm. On the other hand, water-treated glycogen extracted *via* KOH-HW (**Figure 2C,** blue curves) and SDGU-CW (**Figure 2D,** blue curves) exhibits larger particle sizes with peaks at $R_h \sim 40$ nm thus indicating that glycogen α particles are present in the bacteria. Whereas both KOH-HW and SDGU-CW methods extract glycogen α particles, TCA-HW, and TCA-CW methods either degrade larger α particles into β particles or only extract smaller β particles.

In terms of glycogen stability and fragility, DMSO treatment did not alter weight distributions of glycogen particles extracted through TCA-HW (**Figure 2A,** red curves), TCA-CW (**Figure 2B,** red curves), and KOH-HW (**Figure 2C,** red curves). However, glycogen extracted *via* the KOH-HW method mainly consisted of stable α particles while TCA methods mainly generated β particles. As for glycogen extracted from SDGU-CW, a large change of α-particle and β-particle compositions was observed after DMSO treatment. It is inferred from **Figure 2D** that a fraction of the α particles (blue curves) degraded into β particles (red curves), leading to the observed plateau region and thereby suggesting that there is a co-existence of both stable and fragile α particles in bacterial glycogen.

In order to support the morphological structures of glycogen particles extracted from SDGU-CW, representative TEM pictures of glycogen are shown in **Figure 3**. The morphology of raw-state glycogen particles from *E. coli* is similar to that from healthy mouse liver[15], showing the presence of rosette-shaped α particles and few β particles, which confirms that glycogen α particles can be extracted from *E. coli via* mild extraction methods.

Chain-length distributions

The chain length distributions (CLDs) of glycogen particles measured by FACE are shown in **Figure 4**. Average chain length (ACL) is the reciprocal of branching percentage and can be calculated using the formula Σ (DP Percentage x Number of DP). According to the results, glycogen from SDGU-CW (**Figure 4D**) and TCA-CW (**Figure 4B**) had the highest ACLs (~14 DP), indicating that CW extraction had the least CLD damage. For TCA-HW extracted glycogen, chains were partially degraded due to the brief boiling step, as seen from a slight reduction of ACL (**Figure 4A**). Finally, the CLD for glycogen from KOH-HW shifted toward smaller DP values and the ACL decreased by more than 2 DPs (**Figure 4C**), which indicated that boiling in a strongly alkaline medium could damage the primary structure of glycogen particles.

FIGURE AND TABLE LEGENDS:

Figure 1: A schematic illustration of bacterial glycogen extraction through the SDGU-CW method.

Figure 2: SEC weight distributions [*w*(log R_h) (a.u)] for *E. coli* BL21(DE3) glycogen particles extracted through four methods. (**A**) TCA-HW, (**B**) TCA-CW, (**C**) KOH-HW, and (**D**) SDGU-CW. Size distribution curves are shown for glycogen particles treated with water (blue color) and DMSO (red color). All SEC analyses were performed in duplicate. W1: duplicate 1 treated with water. D1: duplicate 1 treated with DMSO. W2: duplicate 2 treated with water. D2: duplicate 2 treated with DMSO. This figure has been reproduced from reference [1].

Figure 3: Representative TEM image. TEM image of bacterial α glycogen particles and β glycogen particles extracted through cold-water extraction using sucrose density gradient ultra-centrifugation (SDGU-CW) method. Representative particles are denoted with red arrows.

Figure 4: Chain length distributions and average chain lengths of *E. coli* glycogen extracted through four different methods. (**A**) TCA-HW, (**B**) TCA-CW, (**C**) KOH-HW, and (**D**) SDGU-CW. Three independent extractions were performed for each method and the average chain length distributions, together with standard error means, were presented. This figure has been adapted from data published in reference [1].

DISCUSSION:

Glycogen is an important energy reserve that has been identified in many bacteria[16]. To dissect the physiological functions of glycogen particles, it is essential to have a better understanding of the fine structure of glycogen molecules. So far, a variety of methods have been developed to extract glycogen from bacterial culture. However, different size distributions of glycogen particles have been observed from different extraction methods, which suggests damaged glycogen structure. Thus, it is necessary to compare and standardize extraction procedures in order to make sure that glycogen structures from different studies are comparable. In this study, a detailed protocol is presented describing four commonly used methods for glycogen extraction from *E. coli* liquid culture, which are then evaluated through structural characterizations of glycogen particles.

As for size distribution, glycogen particles from both TCA-CW and TCA-HW show a weight distribution towards smaller particle size with peaks at $R_h \approx 20$ nm (β particles). Thus, these two methods are not suitable to extract glycogen particles for structural characterization. In fact, it might be part of the reason why only β particles were thought to exist in bacteria since TCA methods have been widely used for bacterial glycogen study. On the other hand, glycogen particles from both KOH-HW and SDGU-CW methods have dominantly larger particle sizes with the highest peaks at $R_h \approx 40$ nm (α particles). Thus, KOH-HW and SDGU-CW methods are better than TCA-CW and TCA-HW methods. However, since only stable α particles can be extracted *via* the KOH-HW method, it indicates that fragile α particles are disrupted by the harsh conditions used in this method.

In terms of chain length distributions (CLD), glycogen from SDGU-CW and TCA-CW have longer chains, which confirms that cold-water extraction results in minimal CLD damage. Chain lengths of glycogen particles extracted *via* TCA-HW were partially reduced due to the brief boiling step in the extraction procedure, leading to a decrement of average chain length (ACL). With the KOH-HW method, CLD reveals shorter chains of glycogen, and the ACL decreases by more than 2 DPs owing to long-term boiling in alkaline solutions. Thus, based on this primary structure analysis of glycogen particles, it is confirmed that cold water extraction, being much milder than hot water extraction, can extract glycogen particles with longer chains and hence less degradation.

In summary, minimal degradation is essential to study the properties of the native glycogen. Minimal degradation of glycogen particles is indicated when size distribution analysis shows larger molecules and chain-length distribution shows the greatest number of longer chains. Since cold water extraction *via* sucrose density gradient ultra-centrifugation achieves the best overall effect from the perspective of glycogen structural integrity, this method is

recommended for bacterial glycogen extraction in future relevant studies.

ACKNOWLEDGEMENTS:

We are greatly thankful to Professor Robert G. Gilbert from the University of Queensland and Yangzhou University who provided insights and expertise that greatly assisted the completion of this study. We acknowledge the financial support of the National Natural Science Foundation of China (No. 31900022, No. 32171281), Natural Science Foundation of Jiangsu Province (No. BK20180997), Young Science and Technology Innovation Team of Xuzhou Medical University (No. TD202001), and Jiangsu Qinglan Project (2020).

DISCLOSURES:

The authors have no conflicts of interest.

REFERENCES:

1 Wang, L. et al. Molecular structure of glycogen in Escherichia coli. *Biomacromolecules.* **20** (7), 2821–2829 (2019).

2 Deng, B. et al. Molecular structure of glycogen in diabetic liver. *Glycoconjugate Journal.* **32** (3–4), 113–118 (2015).

3 Hu, Z. et al. Diurnal changes of glycogen molecular structure in healthy and diabetic mice. *Carbohydrate Polymers.* **185**, 145–152 (2018).

4 Nawaz, A., Zhang, P., Li, E., Gilbert, R. G., Sullivan, M. A. The importance of glycogen molecular structure for blood glucose control. *iScience.* **24** (1), 101953 (2021).

5 Rashid, A. M. et al. Assembly of α-glucan by GlgE and GlgB in mycobacteria and streptomycetes. *Biochemistry.* **55** (23), 3270–3284 (2016).

6 Wang, L. et al. Recent progress in the structure of glycogen serving as a durable energy reserve in bacteria. *World Journal of Microbiology and Biotechnology.* **36** (1), 14 (2020).

7 Kamio, Y., Terawaki, Y., Nakajima, T., Matsuda, K. Structure of glycogen produced by Selenomonas ruminantium. *Agricultural and Biological Chemistry.* **45** (1), 209–216 (1981).

8 Gong, J., Forsberg, C. W. Separation of outer and cytoplasmic membranes of Fibrobacter succinogenes and membrane and glycogen granule locations of glycanases and cellobiase. *Journal of Bacteriology.* **175** (21), 6810–6821 (1993).

9 Mojibi, N. Comparison of methods to assay liver glycogen fractions: the effects of starvation. *Journal of Clinical and Diagnostic Research.* **11**(3), BC17–BC20 (2017).

10 Orrell, S. A., Bueding, E. A comparison of products obtained by various procedures used for the extraction of glycogen. *The Journal of Biological Chemistry.* **239,** 4021–4026 (1964).

11 Tan, X. et al. Proteomic investigation of the binding agent between liver glycogen beta particles. *ACS Omega.* **3** (4), 3640–3645 (2018).

12 Hu, Z. et al. Diurnal changes of glycogen molecular structure in healthy and diabetic mice. *Carbohydrate Polymers.* **185**, 145–152 (2018).

13 Sullivan, M. A., Harcourt, B. E., Xu, P., Forbes, J. M., Gilbert, R. G. Impairment of liver glycogen storage in the db/db animal model of type 2 diabetes: a potential target for future therapeutics? *Current Drug Targets.* **16** (10), 1088–1093 (2015).

14 Deng, B. et al. Molecular structure of glycogen in diabetic liver. *Glycoconjugate Journal.* **32** (3–4), 113–118 (2015).

15 Sullivan, M. A. et al. Molecular structural differences between type-2-diabetic and healthy glycogen. *Biomacromolecules.* **12** (6), 1983–1986 (2011).
16 Wang, L. et al. Systematic analysis of metabolic pathway distributions of bacterial energy reserves. *G3 Genes|Genomes|Genetics.* **9** (8), 2489–2496 (2019).

Figure 1

Figure 2

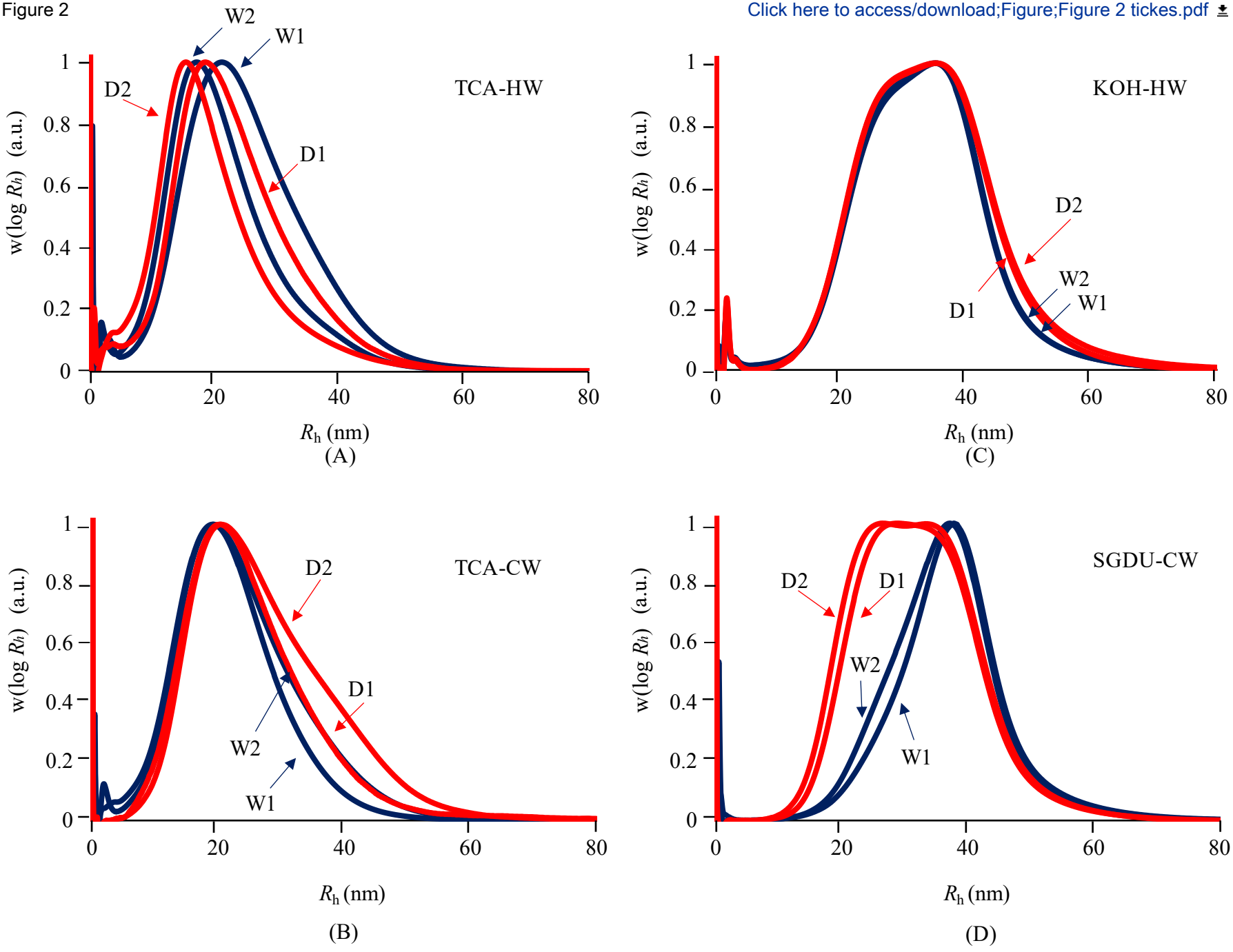


Figure 3

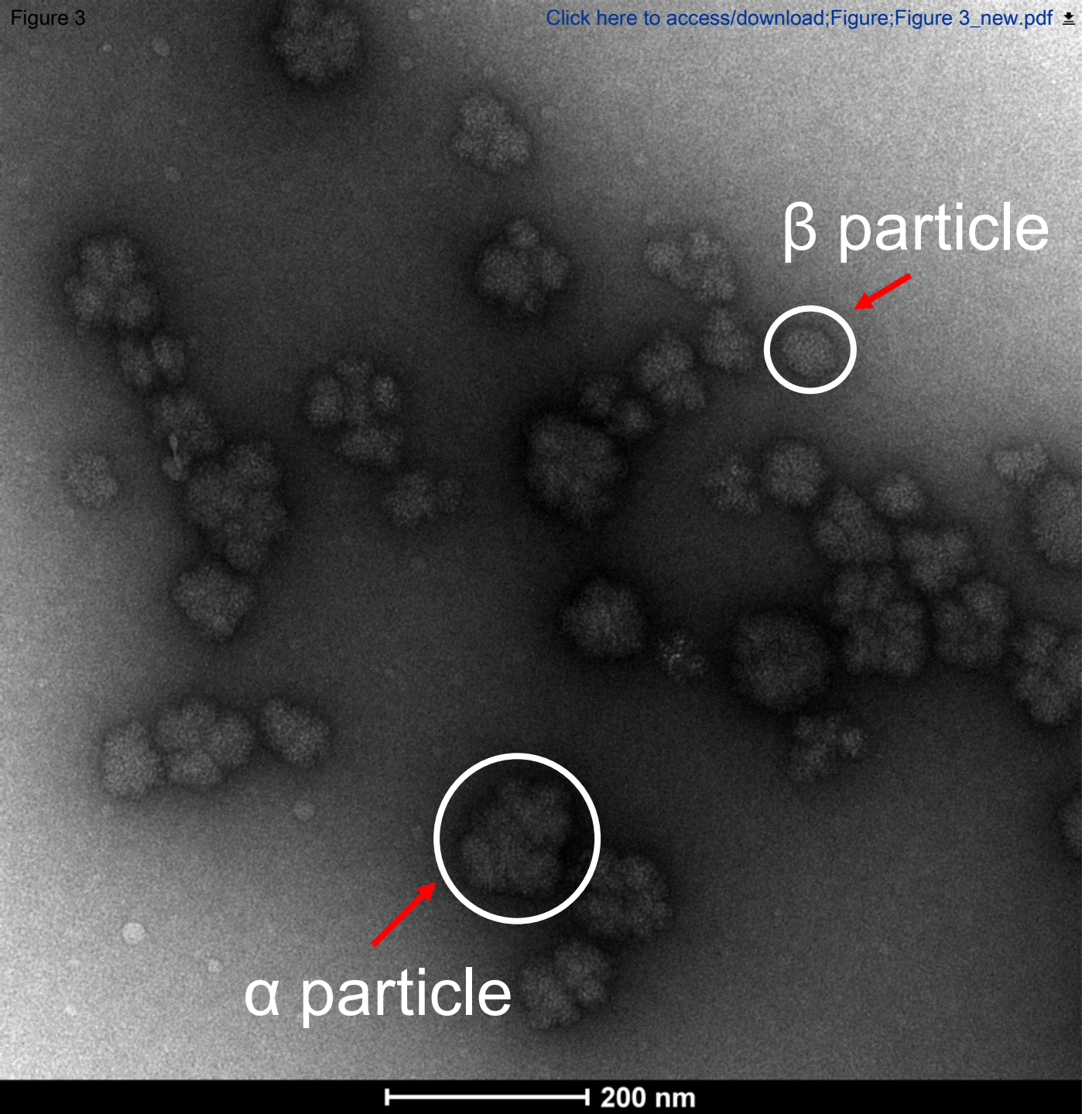
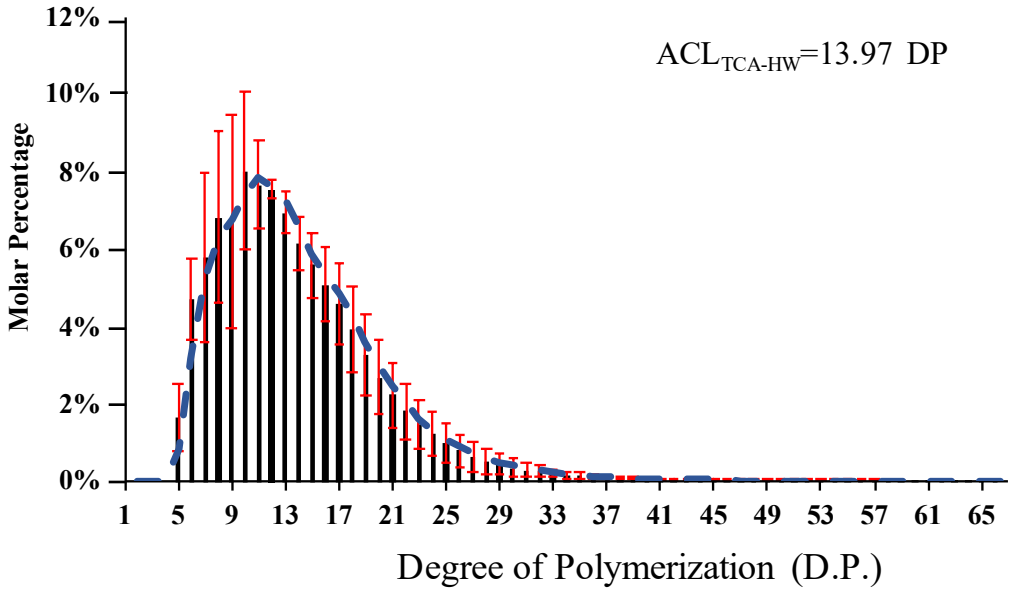
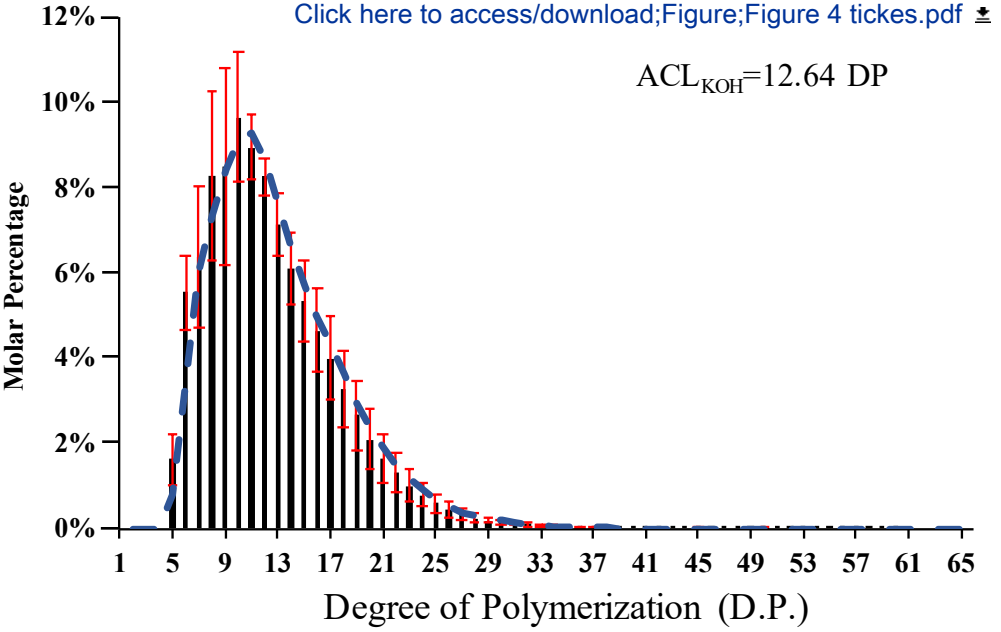


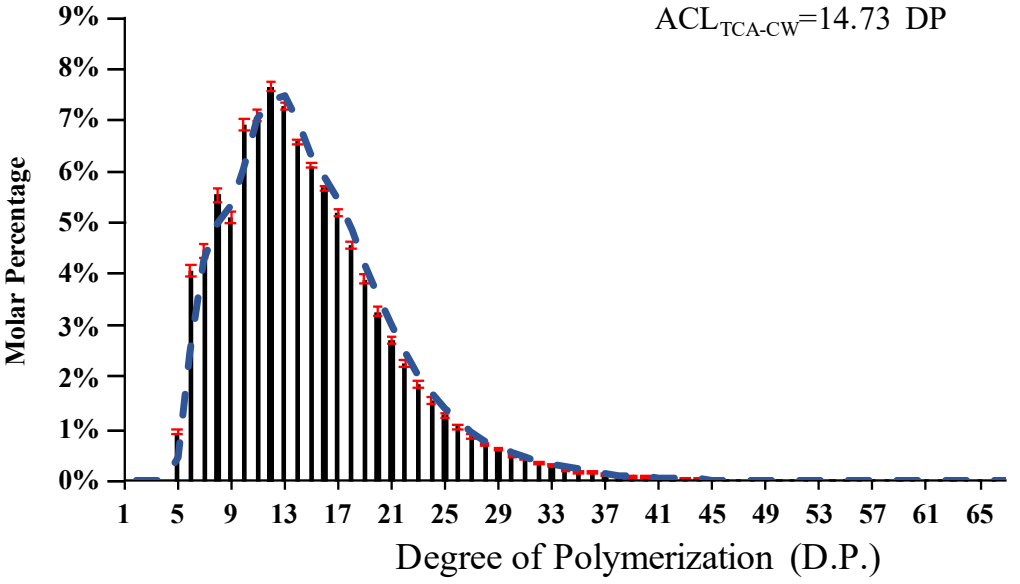
Figure 4



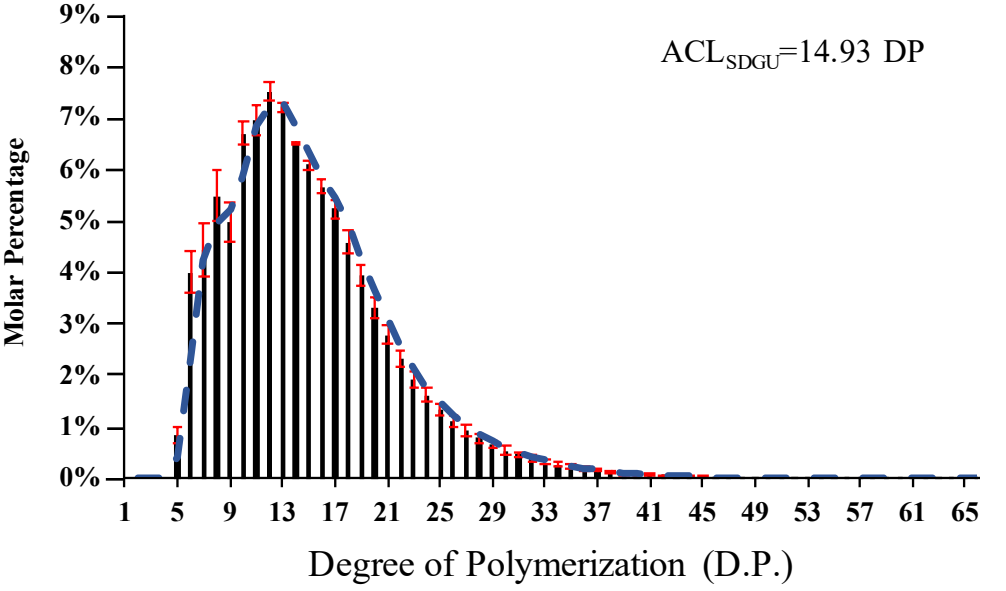
(A)



(C)



(B)



(D)

Click here to access/download
Table of Materials
Table of Materials.xlsx

Responses to Editorial Comments

Ms. Ref. No.: JoVE63016

Date: Dec. 03, 2021

Dear Editorial Office,

We thank the editor for sending us the editorial comments. We have revised the manuscript according to the editors' suggestions. For details, please see the tracked changes in the revised manuscript. We also went through the manuscript and revised wherever applicable. We checked the author's guidelines to make sure that the manuscript sticked to the journal's official formats.

Kind regards,
Liang Wang, PhD

Video Produced by Author: Less than 50 MB. If your video is greater than 50 MB, click "offline" as the delivery method and our

This piece of the submission is being sent via mail.

Dear Dr. Liang Wang,

Thank you for contacting ACS Publications Support.

Your permission requested is granted and there is no fee for this reuse. In your planned reuse, you must cite the ACS article as the source, add this direct link https://pubs.acs.org/doi/10.1021/acs.biomac.9b00586, and include a notice to readers that further permissions related to the material excerpted should be directed to the ACS.

If you need further assistance, please let me know.

Best regards,
Ranjith Alexander
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Ranjith Alexander
ACS Publications Support
Customer Services & Information
Website: https://acs.service-now.com/acs
Email: support@services.acs.org
Phone: 800-227-9919 | 202-872-(HELP) 4357

Case Info:

Case Number : CSCSI0043717

Created On: 12-27-2021 09:03:01 PM EST

Short Description: 回复：RE: [EXT] 回复：Response Requested: JoVE Submission JoVE63016R4 - [EMID:59b3abf98a4e5a92]

Description: Dear ACS Support Team,

We am currently working on a manuscript that is under consideration for publication by the Journal of Visualized Experiments (JOVE). In the manuscript, we would like to reuse the Figure 1 from the published paper (Biomacromolecules 2019, 20, 7, 2821–2829 Publication Date:June 4, 2019, https://doi.org/10.1021/acs.biomac.9b00586) as Figure 2 in JOVE manuscript. In addition, we would also like to re-use the data in Figure 4 from the publish paper (Biomacromolecules 2019, 20, 7, 2821–2829 Publication Date:June 4, 2019, https://doi.org/10.1021/acs.biomac.9b00586) and re-draw it as a new Figure 4 in our JOVE manuscript. For details, please see the attached JOVE manuscript.

We would like to mention that the manuscript (Biomacromolecules 2019, 20, 7, 2821–2829 Publication Date:June 4, 2019, https://doi.org/10.1021/acs.biomac.9b00586) is under the license of ACS
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Author Choice.

We look forward to hearing from you soon.

Best regards,
Liang Wang, PhD

Standard ACS AuthorChoice/Editors' Choice Usage Agreement

This ACS article is provided to You under the terms of this Standard *ACS AuthorChoice/Editors' Choice* usage agreement between You and the American Chemical Society ("ACS"), a federally-chartered nonprofit located at 1155 16th Street NW, Washington DC 20036. Your access and use of this ACS article means that you have accepted and agreed to the Terms and Conditions of this Agreement. ACS and You are collectively referred to in this Agreement as "the Parties").

1. SCOPE OF GRANT

ACS grants You non-exclusive and nontransferable permission to access and use this ACS article subject to the terms and conditions set forth in this Agreement.

2. PERMITTED USES

a. For non-commercial research and education purposes only, You may access, download, copy, display and redistribute articles as well as adapt, translate, text and data mine content contained in articles, subject to the following conditions:

i. The authors' moral right to the integrity of their work under the Berne Convention (Article 6bis) is not compromised.

ii. Where content in the article is identified as belonging to a third party, it is your responsibility to ensure that any reuse complies with copyright policies of the owner.

iii. Copyright notices or the display of unique Digital Object Identifiers (DOI's), ACS or journal logos, bibliographic (e.g. authors, journal, article title, volume, issue, page numbers) or other references to ACS journal titles, web links, and any other journal-specific "branding" or notices that are included in the article or that are provided by the ACS with instructions that such should accompany its display, should not be removed or tampered with in any way. The display of *ACS AuthorChoice* or *ACS Editors' Choice* articles on non-ACS websites must be accompanied by prominently displayed links to the definitive published versions of those articles on the ACS website.

iv. Any adaptations for non-commercial purposes must prominently link to the definitive published version on the ACS website and prominently display the statement: "This is an unofficial adaptation of an article that appeared in an ACS publication. ACS has not endorsed the content of this adaptation or the context of its use."

v. Any translations for non-commercial purposes, for which a prior translation agreement with ACS has not been established, must prominently link to the definitive published version on the ACS website and prominently display the statement: "This is an unofficial translation of an article that appeared in an ACS publication. ACS has not endorsed the content of this translation or the context of its use."

b. Each time You distribute this ACS article or an adaptation, ACS offers to the recipient a license to this ACS article on the same terms and conditions as the license granted to You under this License.

c. For permission to use ACS copyrighted articles beyond that permitted here, visit: http://pubs.acs.org/copyright/permissions.html

3. PROHIBITED USES

a. Use of this ACS article for commercial purposes is prohibited. Examples of such prohibited commercial purposes include but are not limited to:

i. Copying or downloading of articles, or linking to such postings, for further distribution, sale or licensing, for a fee;

ii. Copying, downloading or posting by a site or service that incorporates advertising with such content;

iii. The inclusion or incorporation of article content in other works or services (other than normal quotations with an appropriate citation) that is then available for sale or licensing, for a fee;

iv. Use of articles or article content (other than normal quotations with appropriate citation) by a for-profit organizations for promotional purposes, whether for a fee or otherwise;

v. Sale of translated versions of the article that have not been authorized by license or other permission from the ACS

4. TERMINATION

ACS reserves the right to limit, suspend, or terminate your access to and use of the ACS Publications Division website and/or all ACS articles immediately upon detecting a breach of this License.

5. COPYRIGHTS; OTHER INTELLECTUAL PROPERTY RIGHTS

Except as otherwise specifically noted, ACS is the owner of all right, title and interest in the content of this ACS article, including, without limitations, graphs, charts, tables illustrations, and copyrightable supporting information. This ACS article is protected under the Copyright Laws of the United States Codified in Title 17 of the U.S. Code and subject to the Universal Copyright Convention and the Berne Copyright Convention. You agree not to remove or obscure copyright notices. You acknowledge that You have no claim to ownership of any part of this ACS article or other proprietary information accessed under this Agreement.

The names “American Chemical Society,” “ACS” and the titles of the journals and other ACS products are trademarks of ACS.

6. DISCLAIMER OF WARRANTIES; LIMITATION OF LIABILITY

ACS warrants that it is entitled to grant this Agreement.

EXCEPT AS SET FORTH IN THE PRECEDING SENTENCE, ACS MAKES NO WARRANTY OR REPRESENTATION OF ANY KIND, EXPRESS OR IMPLIED, WITH RESPECT TO THIS ACS ARTICLE INCLUDING, BUT NOT LIMITED TO WARRANTIES AS TO THE ACCURACY OR COMPLETENESS OF THE ACS ARTICLE, ITS QUALITY, ORIGINALITY, SUITABILITY, SEARCHABILITY, OPERATION, PERFORMANCE, COMPLIANCE WITH ANY COMPUTATIONAL PROCESS, MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE.

ACS SHALL NOT BE LIABLE FOR: EXEMPLARY, SPECIAL, INDIRECT, INCIDENTAL, CONSEQUENTIAL OR OTHER DAMAGES ARISING OUT OF OR IN CONNECTION WITH THE AGREEMENT GRANTED HEREUNDER, THE USE OR INABILITY TO USE ANY ACS PRODUCT, ACS'S PERFORMANCE UNDER THIS AGREEMENT, TERMINATION OF THIS AGREEMENT BY ACS OR THE LOSS OF DATA, BUSINESS OR GOODWILL EVEN IF ACS IS ADVISED OR AWARE OF THE POSSIBILITY OF SUCH DAMAGES. IN NO EVENT SHALL THE TOTAL AGGREGATE LIABILITY OF ACS OUT OF ANY BREACH OR TERMINATION OF THIS AGREEMENT EXCEED THE TOTAL AMOUNT PAID BY YOU TO ACS FOR ACCESS TO THIS ACS ARTICLE FOR THE CURRENT YEAR IN WHICH SUCH CLAIM, LOSS OR DAMAGE OCCURRED, WHETHER IN CONTRACT, TORT OR OTHERWISE, INCLUDING, WITHOUT LIMITATION, DUE TO NEGLIGENCE.

The foregoing limitations and exclusions of certain damages shall apply regardless of the success or effectiveness of other remedies. No claim may be made against ACS unless suit is filed within one (1) year after the event giving rise to the claim.

7. GENERAL

This Agreement sets forth the entire understanding of the Parties. The validity, construction and performance of this Agreement shall be governed by and construed in accordance with the laws of the District of Columbia, USA without reference to its conflicts of laws principles. You acknowledge that the delivery of the ACS article will occur in the District of Columbia, USA. You shall pay any taxes lawfully due from it, other than taxes on ACS's net income, arising out of your use of this ACS article and/or other rights granted under this Agreement. You may not assign or transfer its rights under this Agreement without the express written consent of ACS.

8. ACCEPTANCE

You warrant that You have read, understand, and accept the terms and conditions of this Agreement. ACS reserves the right to modify this Agreement at any time by posting the modified terms and conditions on the ACS Publications Web site. Any use of this ACS article after such posting shall constitute acceptance of the terms and conditions as modified.

BioMACROMOLECULES

Article

Cite This: *Biomacromolecules* 2019, 20, 2821−2829

pubs.acs.org/Biomac

Molecular Structure of Glycogen in *Escherichia coli*

Liang Wang,[†,‡,¶] Qinghua Liu,[†,§,¶] Xinle Tan,[∥,⊥] Ziyi Wang,[∥,⊥] Mengmeng Wang,[‡,§] Michael J. Wise,[#] Chengcheng Li,[▽] Chao Ma,[†] Enpeng Li,[○] Bin Deng,[□] Yan Du,[‡,§] Daoquan Tang,*[,‡,§] and Robert G. Gilbert*[,∥,⊥,○]

[†]Department of Bioinformatics, School of Medical Informatics, Xuzhou Medical University, Xuzhou 221000, Jiangsu Province, China

[‡]Jiangsu Provincial Key Laboratory of New Drug Research and Clinical Pharmacy, School of Pharmacy, Xuzhou Medical University, Xuzhou 221000, Jiangsu Province, China

[§]Department of Pharmaceutical Analysis, School of Pharmacy, Xuzhou Medical University, Xuzhou 221000, Jiangsu Province, China

[∥]School of Chemistry and Molecular Biosciences, The University of Queensland, Brisbane, Queensland 4072, Australia

[⊥]Centre for Nutrition and Food Sciences, Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, Brisbane, Queensland 4072, Australia

[#]The Marshall Center for Infectious Diseases Research and Training, and Department of Computer Science and Software Engineering, University of Western Australia, Perth, Western Australia 6009, Australia

[▽]School of Bioengineering, Jiangnan University, Wuxi 214122, Jiangsu Province, China

[○]Joint International Research Laboratory of Agriculture and Agri-Product Safety, College of Agriculture, Yangzhou University, Yangzhou 225009, Jiangsu Province, China

[□]Department of Pharmacy, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei 430030, China

Supporting Information

ABSTRACT: Glycogen, a randomly branched glucose polymer, provides energy storage in organisms. It forms small β particles which in animals bind to form composite α particles, which give better glucose release. Simulations imply β particle size is controlled only by activities and sizes of glycogen biosynthetic enzymes and sizes of polymer chains. Thus, storing more glucose requires forming more β particles, which are expected to sometimes form α particles. No α particles have been reported in bacteria, but the extraction techniques might have caused degradation. Using milder glycogen extraction techniques on *Escherichia coli*, transmission electron microscopy and size-exclusion chromatography showed α particles, consistent with this hypothesis for α-particle formation. Molecular density and size distributions show similarities with animal glycogen, despite very different metabolic processes. These general polymer constraints are such that any organism which needs to store and then release glucose will have similar α and β particle structures: a type of convergent evolution.