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Impact of Temperature Variations on the Entrapment of Bacterial Endotoxins in Aqueous Solutions of Four-Antennary Oligoglycines

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Abstract: Specific self-assembly is registered in aqueous solution formulations based on four-antennary oligoglycines (T4), namely a spontaneous onset of highly ordered nanostructures—tectomers. This phenomenon is initiated by the action of hydrogen-bonding interactions that promote molecular recognition propensities involving Polyglycine-II-type non-canonical architecture. The result is the formation of positively charged supramolecular entities. These have high potential to capture bacterial endotoxins, like lipopolysaccharides (LPSs). By now, it has been established that the overall properties of these systems can be precisely regulated and gradually changed through fine-tuning the parameters in the aqueous environment (composition, pH, etc.). One unexplored option is to clarify the impact of temperature variations. The aim of the present study is to implement systematic investigations on how changes in temperature influence the various options for the removal of trace LPS quantities, captured by the T4 tectomers. The additional goal is to verify the possibility to develop consecutive paths of recovering the extra T4 quantities that have not participated in the formation of T4+LPS complexes. Some prospects for further applications, e.g., in medicine and pharmaceutics, are also generally outlined.

Keywords: four-antennary oligoglycines; tectomers; lipopolysaccharides; surface tension; surface dilational rheology

Citation: Gyurova, A.Y.; Arabadzhieva, D.; Minkov, I.; Nikolov, L.; Mileva, E. Impact of Temperature Variations on the Entrapment of Bacterial Endotoxins in Aqueous Solutions of Four-Antennary Oligoglycines. *Colloids Interfaces* **2023**, *7*, 62. https://doi.org/10.3390/colloids7040062

Academic Editors: Aleksandra Szcześ, Margaritis Kostoglou and Reinhard Miller

Received: 25 August 2023
Revised: 30 September 2023
Accepted: 8 October 2023
Published: 11 October 2023

1. Introduction

Four-antennary oligoglycines ($[Gly_n\text{-}NHCH_2]_4C$, T4) are synthetic substances with specific self-assembling performance in aqueous environments [1,2]. Due to the presence of equal-length oligopeptide tails attached to a central carbon atom, they can build up disk-like supramolecular entities—*tectomers*. The key driving force for the onset of these nanostructures is the predisposition of T4 molecules to form extended intra- and intermolecular H-bonding networks at high cooperativity levels arranged in Polyglycine II motifs (PG-II, helix-3_1) [3–5]. Because of the four-tail bonding to a central C atom, appropriate mutual orientations of CO and NH groups in the neighboring antennae within a single oligoglycine molecule are possible, which result in the onset of intramolecular helix-3_1 motifs. This inherent PG-II tendency generates the inception of intramolecular "click-clack" configurations and creates a specific *H*-conformation of the single T4 molecule [1,2]. These primarily structured species serve as building blocks for the further development of the tectomeric self-assemblies. These are based on the consecutive association of *H*-formed T4 units via intermolecular H-bonds into hexagonal packing of disk-like supramolecular entities, characterized by unusual stability [1]. This phenomenon is closely related to the number of the glycine units in the antennae: there must be at least 7 ($n \geq 7$) so as to fulfill the

demands of configuring the effective "click-clack" network of highly coordinated intra- and intermolecular helix-3_1 motifs [1].

In fluid environments, the onset of PG-II tendencies is coupled with auxiliary interaction options involving the other components of the system. For example, in aqueous solutions and at ambient conditions, portions of NH_2 terminal groups are charged to NH_3^+ [1,2,6], and both the single oligoglycine molecules and the tectomers are positively charged in water. Hence, complementary fine-tuning possibilities for changes in the structure–property relationships arise, e.g., through variations in pH, the addition of low-molecular-mass (LMM) electrolytes, and the insertion of negatively charged ingredients. The interplay of the PG-II-formation tendencies and the aqueous solution composition provides considerable application potential for solutions containing four-antennary oligopeptides [2,6]. An essential option has been identified recently: in the presence of traces of bacterial lipopolysaccharides (LPS, endotoxins), T4+LPS complexes are formed so as to effectively capture and block the endotoxins in an aqueous environment [6–8].

LPSs build the external cover layer of the cell walls of Gram-negative bacteria like the human pathogen *Escherichia coli* (*E. coli*) [9,10]. When separated from the cell wall, LPSs remain toxic, with the lethal concentrations for humans being very low [11–14]. While there are methods to capture LPSs in aqueous media, they are either too specific or refer to higher quantities of the endotoxin (e.g., [13–15]). The development of new approaches, particularly those aimed at the identification of ultralow quantities of LPSs, are constantly needed, and one such approach was proposed in our earlier studies [6–8]. It is based on the fact that LPS molecules are amphiphilic substances: as key structural components, they have hydrocarbon chains (Lipid A) and a core polysaccharide region. In particular, the presence of phosphate groups in the core portions is responsible for the electric (negative) charge of the LPS in an aqueous environment. Due to electrostatic interactions with the T4 supramolecular entities in water solutions, two major types of T4+LPS complexes appear: amphiphilic species with the LPS molecules attached to one site of the tectomers, and "sandwich-like" hydrophilic T4+LPS entities, with LPS in between two discotic T4 self-assemblies [6,8]. At sufficient T4 content in the in aqueous milieu, these phenomena result in complete entrapment of the LPS traces. In addition, the onset of the T4+LPS complexes and their sizes may be additionally regulated via changes in the pH of the systems at ambient temperatures [8]. An important unsolved problem remains, however: how to remove the entrapped LPS from the aqueous solutions. This issue is particularly important in cases where super-clean water is necessary, e.g., in hemodialysis, neonatology, etc.

The aim of the present study is to implement systematic investigations of the influence of temperature variations on the different options for removal of trace LPS quantities captured by the T4 tectomers. One additional goal is also to verify the possibility to develop consecutive recovering of the extra T4 quantities that did not participate in the formation of T4+LPS complexes. This research concerns both bulk solution structure–property performance and adsorption-layer enactment as a result of moderate changes in temperature in aqueous systems.

2. Materials and Methods

Four-antennary oligoglycine (T4) was purchased from PlasmaChem GmbH (Berlin, Germany) in a salt form ($[Gly_7\text{-}NHCH_2]_4C^*4HCl$, purity 95%). Lipopolysaccharides from *Escherichia coli* EH100 (LPSs) were supplied by Sigma Aldrich (Ra-mutant, purity $\geq$ 97%). Doubly distilled water (2D water) was used for the preparation of the aqueous samples. The T4 solutions were initially incubated for 24 h. The oligoglycine concentration was 2×10^{-4} mol/L. The LPS solutions were prepared with an initial concentration of 5 μg/L and stored at 4 °C for at least one month. The investigated fluid systems were obtained from the LPS stock solution and the final endotoxin concentration in all measurements was 0.5 μg/L. The specific choice of the concentrations corresponds to the optimal T4 quantity for capturing the whole LPS quantity at the "native pH" (pH = 5.70 at 20 °C), as established in [8].

Britton–Robinson universal buffer was applied to maintain the pH of the samples [16]. The buffer was composed of boric acid (H_3BO_3, Sigma Aldrich, St. Louis, MO, USA, purity $\geq$ 99.97% (trace metal basis)), phosphoric acid (H_3PO_4, Sigma Aldrich, bpurity $\geq$ 99.999% (trace metal basis)), and acetic acid (CH_3COOH, Sigma Aldrich, purity $\geq$ 99.99% (trace metal basis)) in the ratio of 1:1:1 by volume. The components were mixed with sodium hydroxide (Sigma Aldrich, NaOH, purity $\geq$ 97% (titration using HCl)) so as to obtain a fixed pH = 6.2.

The adsorption layer properties at the air–solution interface were investigated by means of the emerging-bubble option of profile analysis tensiometry (PAT-1, Sinterface, Berlin, Germany) [17–19]. The measurements were implemented in the course of about 48 h. The surface dilational rheology was studied through oscillations at low frequencies of 0.005–0.2 Hz; the oscillation amplitudes were in the range of 5% of the bubble surface area. The experiments were carried out at various temperatures in between 20.0 $\pm$ 0.1 and 85.0 $\pm$ 0.1 °C, with the samples kept either in the thermostatic chamber of the PAT instrumentation or in another thermostatic device between the separate measurements.

Some test examinations of the bulk solution properties were performed via dynamic light sScattering (DLS, Zetasizer Nano ZS, Malvern Instruments Ltd., Malvern, UK). The scattering angle was 173° (non-invasive backwards scattering). The aggregate size distributions were recalculated using Mie theory [20,21].

Two types of systems were investigated:

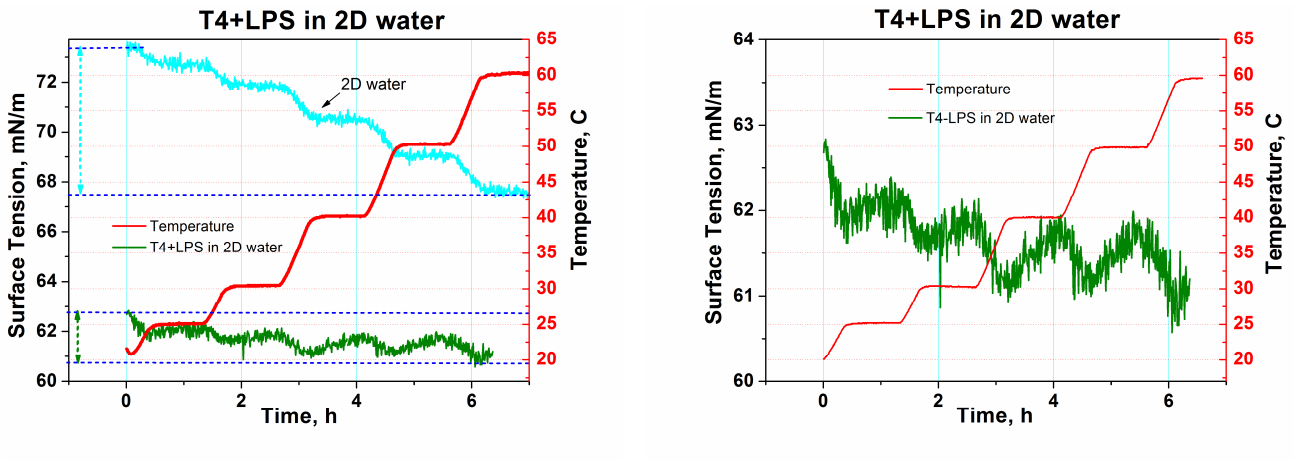
(1) Aqueous solutions of T4+LPS, prepared with doubly distilled water (2D water), at "native pH", without specific pH regulation (pH = 5.7 at 20 °C). This procedure included initial thermal equilibration for 20 min. The temperature was further increased stepwise from 20 °C to 60 °C at ten-degree intervals, keeping the system for the respective thermal equilibration in the course of 1 h at all the thermal steps. The surface dilational rheology was investigated thereafter using the same bubble. Finally, the system was left at 60 °C in the course of 7 h, then subjected to a sharp cool-down procedure back to 20 °C.

(2) Aqueous solutions of T4+LPS, and of T4 only, prepared with Britton–Robinson universal buffer, at specific pH and ambient temperature (pH = 6.2 at 20 °C). These values were chosen because in previous studies it was established that at these conditions, the whole quantity of the LPS traces in the system is evidenced to be captured by the tectomers [8]. The system was exposed to a ten-degree step-wise heating procedure, the same as in case (1). The solution was kept for 7 h at 60 °C, thereafter followed by a slow cooling process, with one-hour steps down to 20 °C. The latter temperature was maintained for the course of 3.5 h, and the last cooling temperature step was down to 10 °C, which was also kept constant for 3.5 h. Another temperature heating–cooling cycle was performed in the range of 20 °C to 85 °C, with various times of incubation at 85 °C (30 min, 1 h, 4 h), followed by either a fast or slow (24 h) cooling procedure down to 20 °C.

3. Results

3.1. Aqueous Solution of T4+LPS at "Native pH"

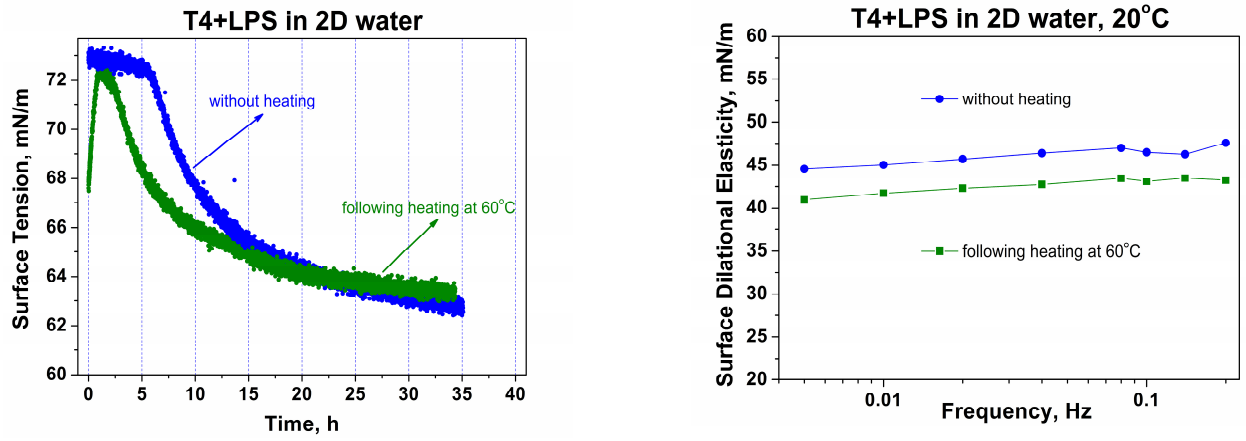
The runs of the dynamic surface tension results of the pure 2D-water system and of T4+LPS systems at "native pH" in the range of 20 °C to 60 °C, are presented in Figure 1a. Note that the values for water are in accordance with well-known published data, e.g., [22]. A more detailed examination of the outcomes reveals the following peculiarities: (i) The overall change in surface tension values is ~1 mN/m for the T4+LPS solution; this is in contrast with the drop of $\geq$5 mN/m for 2D pure water. (ii) While the increase in temperature produces an initial decrease in the surface tension as expected, keeping the attained temperature in the course of one hour results in a somewhat reversed run of the surface tension for the T4+LPS solution, striving to recover the primary value at the previous temperature step (Figure 1b); this tendency is intensified at higher temperatures. These outcomes evidence the stable presence of amphiphilic entities in the T4+LPS samples during the heating–cooling cycle.



(**a**) (**b**)

Figure 1. (**a**) Run of dynamic surface tension vs. time for 2D water and for aqueous solution of T4+LPS at "native pH". The temperature is increased stepwise in the range of 20 °C to 60 °C. (**b**) Detailed view of the results for aqueous solution of T4+LPS at "native pH" (pH = 5.7).

Reaching the temperature of 60 °C, it is retained constantly for 7 h. Thereafter, the system is subjected to strident cool-down to 20 °C; the outcome is presented in Figure 2a. One interesting feature is that at the initial abrupt decrease in temperature to 20 °C, the surface tension values increase sharply up to 72.4 mN/m. Further on, keeping the system at 20 °C in the course of hours results in a full recovering of the initial values characteristic for the system without performing the heating procedure. This outcome might be interpreted as possible structural reorganizations in the heated sample, which are reversible to a certain extent.



(**a**) (**b**)

Figure 2. Adsorption layer properties at air–solution interface at 20 °C and "native pH", (pH = 5.7), without heating and following a sharp decrease in temperature from 60 °C to 20 °C. (**a**) Dynamic surface tension vs. time. (**b**) Surface dilational elasticity vs. frequency.

In order to check the latter notion surface, dilational elasticities were obtained. The values, however, remain systematically lower for the solution disposed to the temperature variations as compared to the non-heated solution for all imposed frequencies of oscillations. (Figure 2b). These data suggest an increase in the number of the surface active species as a result of heating the samples.

3.2. Aqueous Solution of T4+LPS at Controlled pH Value

The experimental results from the stepwise heating and cooling procedure of the aqueous solution at controlled pH = 6.2 are shown in Figure 3. The system was kept for 30 min at the first two temperature steps (20 °C and 30 °C), followed by consecutive preservation of every acquired temperature for one hour. Again, like the experiments in Section 3.1, after reaching 60 °C, the thermal conditions were retained for 7 h. The decreasing-temperature procedure comprises one-hour steps up to the attainment of 20 °C. Thereafter, at 20 °C and 10 °C, the samples were kept for 3.5 h at each step (Figure 3).

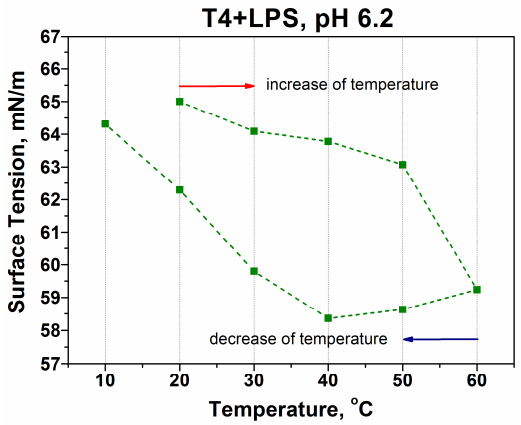
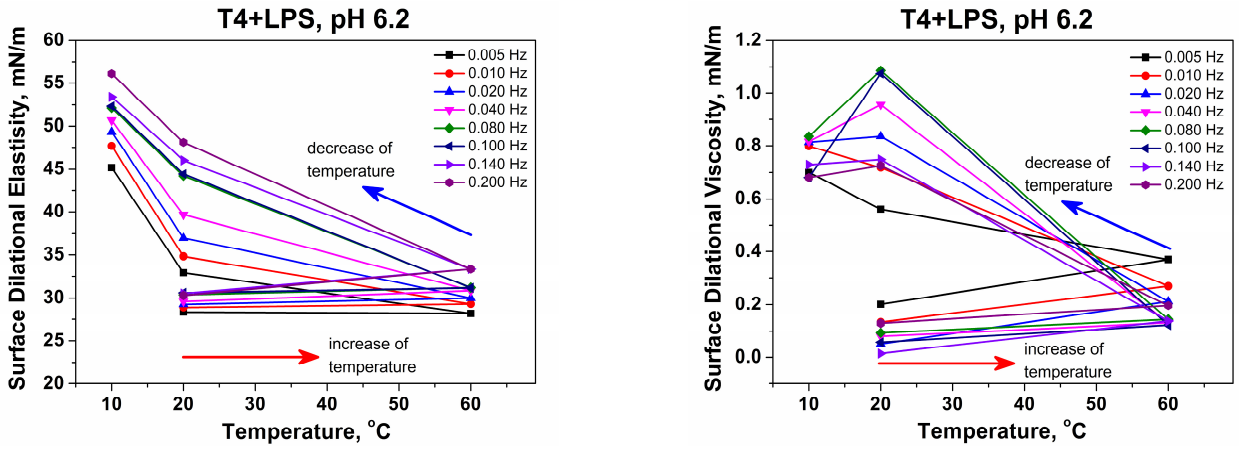


Figure 3. Dynamic surface tension vs. temperature as it is increased and decreased stepwise in the range of 20 °C to 60 °C for aqueous solution of T4+LPS at controlled pH = 6.2.

An unexpected result was observed, namely, the overall surface tension values still slightly decrease, reaching a minimum at 40 °C, then go up with the further drop in temperature. This evidences a tendency of delayed structure–property changes during the temperature variations. The most remarkable outcome is the sensible difference of surface tension values for the same degrees in the heating and the cooling paths, forming a well-expressed hysteresis loop. The latter is indicative of irreversible structural reorganizations in the system throughout the heating–cooling procedure.

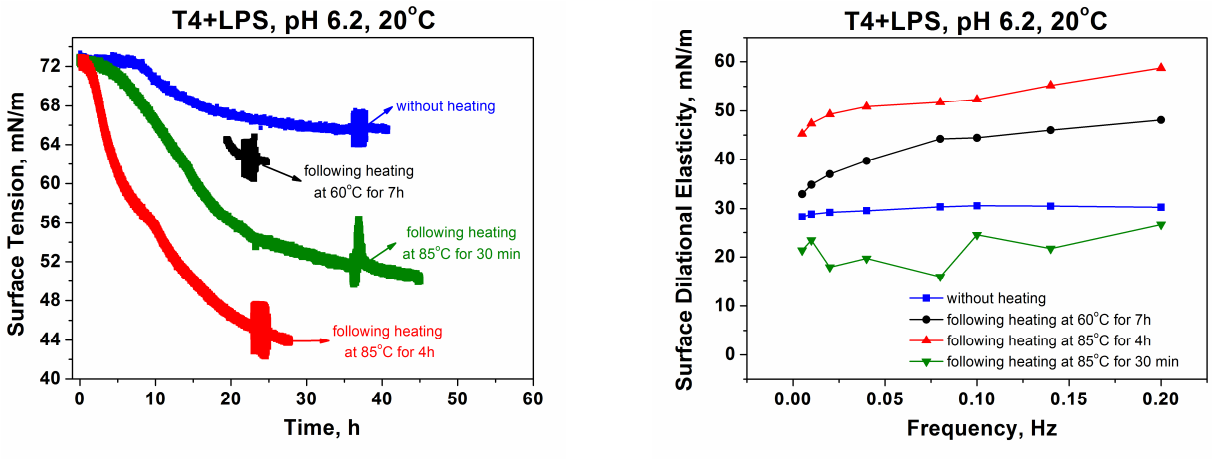
The runs of the surface rheology data also reveal interesting tendencies. In the increasing-temperature branches of the relationship, only slight changes (increase) are observed (Figure 4a,b). In the decreasing-temperature portion of the data, however, a sharp increase in the surface dilational elasticities is registered (Figure 4a). The same trend is observed in the surface dilational viscosities as well; they are more enhanced in the decreasing-temperature steps, compared to the insignificant rise in the heating route (Figure 4b).

Some test experiments were also performed, with heating to temperatures of 85 °C. The obtained data are presented in Figure 5a,b. The system was then kept either for 30 min, 1 h, or 4 h at 85 °C before performing the cooling procedure. As can be seen, with keeping the mixed T4+LPS system in the course of 4 h at 85 °C, a sharper decrease in the surface tension following the cooling procedure to 20 °C is registered, and considerably lower final surface-tension values are reached, as compared to the case of a system initially retained for 30 min at 85 °C and also juxtaposed with a dynamic surface tension run in the samples without heating (Figure 5a). Some of the results for 20 °C, obtained following heating to temperatures of 60 °C and consecutive cooling according to the already described procedure (see Figure 3), are included in Figure 5a for comparison.



(**a**) (**b**)

Figure 4. Surface dilational rheology upon increase and decrease in temperature in the range of 20 °C to 60 °C. (**a**) Surface dilational elasticity at various frequencies. (**b**) Surface dilational viscosity at various frequencies.



(**a**) (**b**)

Figure 5. (**a**) Dynamic surface tension vs. time at 20 °C following heating and cooling procedures at various temperatures. (**b**) The respective changes in the surface dilational elasticity vs. frequency.

The surface dilational elasticity data following heating are considerably higher than the outcomes at lower temperatures (Figure 5b). However, at the end of the cooling procedure, the longer time of the heating (4 h at 85 °C) resulted in considerably lower surface dilational elasticities, as compared to outcomes of the short-time heating procedure (30 min at 85 °C). Note that these data also lay lower than both of the outcomes following heating in the course of 7 h at 60 °C and the surface dilational elasticities without heating the sample (20 °C). These effects might be interpreted as proof of more intense structural changes upon temperature variations, both in the bulk of the solution and at the air–solution interface, during the longer-time-heating event.

4. Discussion

The obtained results might be summarized as follows:

1. The combined heating-and-cooling cycle results are dependent on the pH of the aqueous solutions.

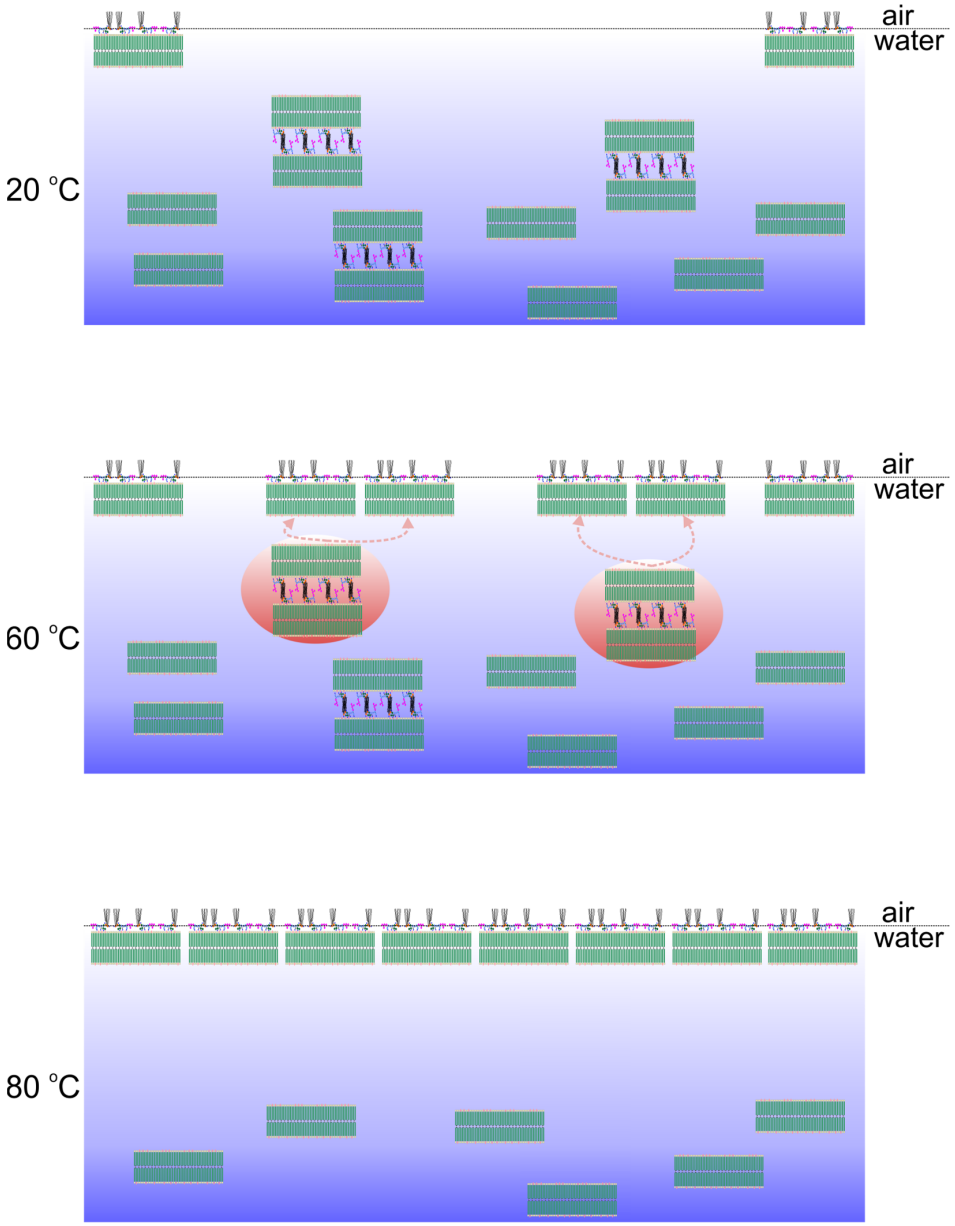
At "native pH" (pH = 5.7), the initial (20 °C, at the start of the heating procedure) surface-tension values are recovered upon proceeding back to 20 °C, while their time evolution differs: there is a visible "lag-period" in the case without heating and a sharp decrease upon cooling the heated sample (Figure 2a). However, at the end of the heating–cooling cycle, the surface-dilational elasticities are systematically lower compared to the respective data for the unheated samples (Figure 2b). The results might be interpreted as being due to an onset of structural reorganizations in the sub-interfacial bulk region that are still not so advanced enough as to have an impact on the equilibrium surface tension of the samples.

At the end of the heating–cooling procedure at controlled pH = 6.2, lower surface tensions (Figures 3 and 5a) and higher surface dilational elasticities (Figures 4a and 5b) are achieved. The higher the temperatures reached upon heating the sample, the lower the final surface tension values obtained at the end of the heating–cooling cycle (20 °C). The data strongly evidence that upon temperature variations, the structural characteristics, both in the subsurface bulk phase and at the adsorption layer at the air–solution interface, are irreversibly changed. In addition, the results in Figure 4a,b evidence that in the heating branch, the possible structural changes are mainly in the solution bulk, while in the cooling branch, we do already have a depletion of the amphiphilic species, most probably due to their adsorption at the air–solution interface within the time of keeping the system at 60 °C.

2. The runs of the surface tension data at controlled pH = 6.2 and 20 °C are very sensitive to the time variations in preliminarily keeping the system at a particular high temperature (Figure 5a). The longer incubation time of the samples at high temperature results in a sharper decrease in the surface tension values and lower final data when cooled to 20 °C. The respective changes in the surface dilational elasticities are even more affected by both the temperature changes and the duration of keeping the samples at high temperatures (Figure 5b). These facts also indicate that the acquired surface tension values and the surface rheological data are actually quasi-stationary and depend on the incubation time at the specific system's conditions. The detailed procedure of the temperature-decrease route and the onset of the well-outlined hysteresis loop additionally demonstrate a clear coupling between the kinetics of structural reorganization in the investigated systems and the temperature variations (Figure 3).

The obtained experimental outcomes give grounds for the advancement of the following hypothesis:

Due to the specific interrelationships between the PG-II tendencies and the components of the surrounding aqueous environment, the structural evolution of the two types of T4+LPS complexes, namely amphiphilic and "sandwich-like" entities, can be effectively regulated through temperature variations and the duration of the incubation times at the highest temperature values. The data evidence that upon heating, the sandwich-like species are "opened"/destroyed, becoming amphiphilic species (Scheme 1). This is fully in line with classical concepts about temperature-variation effects on hydrophobic interactions [23,24]. Upon heating the samples, the impact of thermal regulation results in the weakening of the H-bonded network in aqueous solution. Thus, the "sandwich"-type T4+LPS complexes that are formed because of hydrophobic interactions LPS+LPS at low temperature (20 °C) are "loosened" at higher temperatures (60 °C and 85 °C) and turned into amphiphilic T4+LPS complexes. Due to the innate amphiphilicity of the LPS molecules, these T4+LPS species are surface-active and effectively adsorb at the air–solution interface (Scheme 1). Therefore, the recovery to the initial sandwich-entities is actually impossible/incomplete at the cooling back to ambient temperature (20 °C) in the samples with air–solution interfaces. The higher the heating temperature (85 °C as juxtaposed to 60 °C), and the longer the incubation time at the highest temperatures of the respective thermal cycle (30 min as juxtaposed to 4 h), the more expressed this effect (see Figure 5a,b).



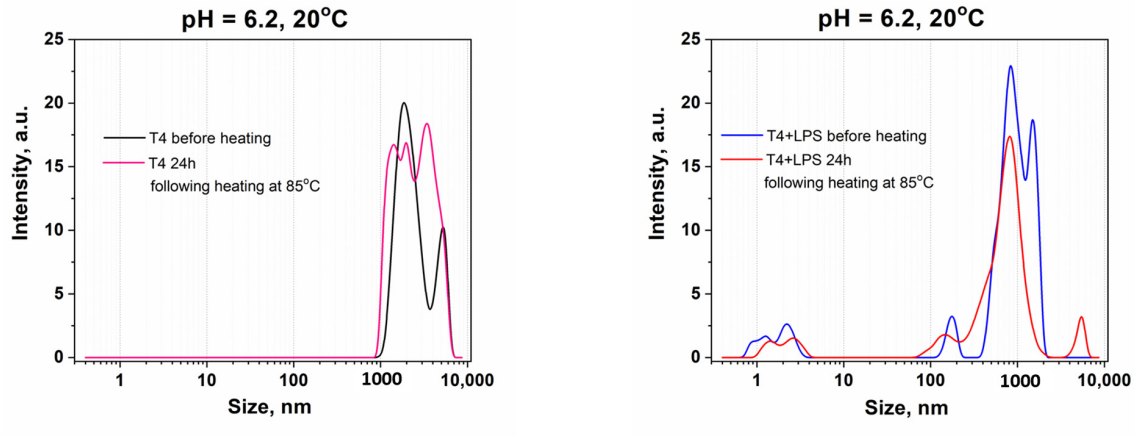
Scheme 1. A sketch of the structural reorganization of the T4+LPS complexes in the aqueous solution bulk and at the air–solution interface upon increase in the temperature.

So, it might be expected that in the particular aqueous solution formulation (C(T4) = 2 × 10^{-4} mol/L; C(LPS) = 0.5 μg/L) at the highest investigated temperature (~80 °C) and under controlled pH = 6.2, the whole quantity of the LPS is caught in amphiphilic T4+LPS complexes. Most probably, the remaining hydrophilic tectomers are not substantially restructured in these conditions because of the PG-II-driven "click-clack" structural portions, and they remain in the bulk of the aqueous solution.

This hypothesis is still preliminary and needs further systematic investigations and should be further verified through other techniques, e.g., X-rays and neutron scattering. However, it is additionally supported by some test DLS measurements (Figure 6).

The DLS data verify that there is no substantial change in the bulk size distribution of the structural entities before and after the heating–cooling cycle, both for the T4-only and for the T4+LPS aqueous solutions, at the temperature of 20 °C. Similarly to the case of ambient conditions [8], the intensity is determined mainly by the size distribution of the tectomeric aggregates. The registered slight shift to larger sizes in the T4 case might

be related to the more intense movement of the tectomers at higher temperatures, which enhances the possibility of the onset of some larger tectomer species formed by the initially smaller T4 entities in the cooling branch of the procedure. However, additional and more consistent studies are needed to verify such a hypothesis.



(**a**) (**b**)

Figure 6. Mean size distributions by intensity of the scattered light for buffered aqueous solutions. The experiments were performed at a temperature of 20 °C before and after the heating–cooling procedure in the range of 20 °C to 85 °C and pH = 6.2. The initial incubation time of T4 solutions was 24 h and of LPS solutions was 2 months. (**a**) T4 solutions at concentration C(T4) = 2×10^{-4} mol/L; (**b**) mixed solutions of T4 and Ra LPS from *E. coli* EH100 at concentrations C(T4) = 2×10^{-4} mol/L and C(LPS) = 0.5 μg/L.

5. Conclusions

The present study provides clear evidence that the capture and removal of endotoxin traces by tectomers of synthetic four-antennary oligoglycines in aqueous solution formulations can be effectively controlled through temperature variations, the duration of the incubation time at the highest temperature upon heating, and changes in the mode of the cooling procedure (either slow stepwise or sharp temperature decrease). The results open a new application pathway for the capture of trace quantities of endotoxins in aqueous environments through the use of biocompatible and considerably cheap synthetic four-antennary oligopeptides. The key elements of the proposed procedure involve moderate heating of the samples, removal of the endotoxins being captured by the tectomers, and consecutive release of the unused T4 quantities. In combination with previous data about the options of pH regulation of T4+LPS water solutions [8], this investigation opens new possibilities to develop nanostructured sensors for the testing and verification of super-cleanness for waters to be used in hemodialysis, neonatology, pharmacology, drug delivery, etc.

Author Contributions: Conceptualization, E.M.; methodology, E.M., D.A., I.M. and A.Y.G.; formal analysis, A.Y.G., L.N. and D.A.; investigation, A.Y.G., D.A. and I.M.; resources, E.M.; data curation, E.M. and L.N.; writing—original draft preparation, D.A. and E.M.; writing—review and editing, E.M. and L.N.; visualization, D.A., L.N. and A.Y.G.; supervision, E.M.; project administration, A.Y.G. and D.A.; funding acquisition, E.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Bulgarian National Fund for Scientific Research through Project "Design and Characterization of Soft Nanostructured Materials Based on Antennary Oligoglycines", grant number КП-06-Н39/5.

Data Availability Statement: Data are available upon request.

Conflicts of Interest: The authors declare no conflict of interest.

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