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Insights on the Structure, Molecular Weight, and Activity of an Antibacterial Protein-Polymer Hybrid

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Abstract

Protein-polymer conjugates are attractive biomaterials which combine the functions of both proteins and polymers. The bioactivity of these hybrid materials, however, is often reduced upon the conjugation. It is important to determine and monitor the protein structure and active site availability, in order to optimize the polymer composition, attachment point, and abundance. The challenges in probing these insights are the large size and high complexity in the conjugates. Here, we overcome the challenges by combining Electron Paramagnetic Resonance (EPR) spectroscopy and Atomic Force Microscopy (AFM) and characterize the structural insights of antibacterial hybrids formed by polyethylene glycol (PEG) and an antibacterial protein. We discovered that the primary reasons for activity loss were PEG blocking the substrate access pathway and/or altering protein surface charges. Our data indicated that the polymers tended to stay away from the protein surface and form a coiled conformation. The structural insights are meaningful for and applicable to the rational design of future hybrids.

Keywords

AFM; Biophysics; EPR spectroscopy; PEGylated protein; structure-activity relationship

Introduction

Main Text Paragraph Protein-polymer hybrid molecules are inspiring biomaterials because they retain the functions/properties of both protein and polymeric materials.^[1–6] For example, upon attachment of neutral polymers, the host protein often shows enhanced stability in the biological environment.^[7–10] This is especially useful for protein delivery wherein the protein often experiences varied (and often harsh) biochemical environments.^[11–15] When placing a “smart” polymer close to the active site of a protein, it becomes

possible to control protein function via controlling the polymer function.^[16–18] On the other hand, introducing proteins into polymeric materials provides enhanced biocompatibility and biological functions. Hybrids indeed have found applications in biomolecular delivery,^[19,20] therapeutics,^[2,16,21–24] and antibacterial materials.^[25–27]

In spite of the (potentially) outstanding properties, the hybrid bioactivity is often seriously reduced upon the conjugation of with polymers. Such activity loss limits the broader application and further development of hybridized biomaterials. To overcome this limitation, it is important to know the structural basis of the activity loss (i.e. protein structure perturbation, polymer affecting protein surface properties or substrate access, etc). Furthermore, when attempting to reduce the activity loss, monitoring the structure is important for assessing the impacts of polymer composition, size, attachment point, and abundance on the bioactivity and overall function. This is, however, a nontrivial task, because it requires detailed hybrid structural information, such as 1) the polymer attachment sites and selection of conjugation strategy,^[28–32] 2) the polymer^[33] and protein conformations upon hybridization, and 3) the polymer docking state onto the protein (wrapping around or extending away). Obtaining this information using the current hybrid characterization techniques^[34,35] or traditional structure determination approaches is difficult because of the intrinsic complexity such as the high heterogeneity, large size, and high dynamics of both polymers and proteins. In addition, controlling and optimizing the site(s) and number of polymers attached is challenging. Lastly, measuring the hybrid concentration and molecular weight (m.w.) is often complicated by polymers.

One promising approach to remove these technical barriers is Electron Paramagnetic Resonance (EPR) spectroscopy. EPR has been widely used in probing structure and conformational dynamics in complex, large biological or polymeric systems.^[36–46] Recently, we have reported EPR in probing structural insights at the complex nano-bio interfaces^[47,48] and in polymeric micelles.^[49] EPR can probe structural information in the native state of the target system, regardless of the size and complexity. In addition, once spin labeled, the hybrid's concentration is proportional to the spin concentration which is immune of any complications caused by polymer optical absorption. Lastly, EPR line shape is sensitive to the hydrodynamic radius of the hybrid molecules, which provides an opportunity to estimate hybrid molecular weight.

In this work, we present the synthesis, characterization, and potential application of antibacterial hybrids formed by an antibacterial protein, T4 lysozyme (T4L), and a series of polyethylene glycol (PEG). We selected the antibacterial T4L^[50,51] because of the increasing interest in the development of antibacterial materials^[52] and ease of evaluating the bioactivity.^[52] We first optimized the conjugation strategy and reaction conditions. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) and Atomic Force Microscopy (AFM) were then utilized to confirm the formation of hybrids. The bioactivity was assessed using a commercial kit, wherein various extents of activity loss were observed. To probe the structural basis of the loss we utilized Circular Dichroism (CD) and EPR to probe the secondary and tertiary structural changes upon PEG attachment. In addition, gold nanoparticles (AuNPs) were used to sense the protein surface charge,^[47] and a clear change in T4L surface charge was found. These efforts revealed reasons for hybrid

bioactivity loss and suggested possible polymer conformations in the hybrids. This is, to the best of our knowledge, the first EPR investigation on probing structural information in protein-polymer hybrids. The strategies and methods reported can be applied to other hybrid systems.

Results and Discussion

T4L is a good candidate for our study because of the close relationship of its structure and activity.^[53,54] We select PEG because of its good biocompatibility, neutral surface charge which weakens the electrostatic interactions, and the low size heterogeneity. In fact, PEG has been approved by US Food and Drug Administration and European Medicines Agency for biomedical use. Two common approaches to conjugate polymers with proteins are grafting-to and growing-from.^[4,55–60] We focus on the grafting-to approach so that the need of modification to the host protein is minimal. It is easier to control the polymer chain length of the formed hybrids since commercial polymers often have relatively homogeneous sizes. A drawback of this approach is the relatively low reaction efficiency stemming from the large sizes of both proteins and polymers.^[61] We overcome this barrier by increasing the polymer-to-protein feeding ratios (mole-to-mole ratios).

Polymer modification.

We started with the commercial PEG5k-OH (Mn~5000). This polymer was modified to PEG5k-COOH and PEG5k-NH₂ as described in the Supporting Information (SI). We found that the PEG-COOH is optimal for synthesizing hybrids (see below). Therefore, similar routes were used to generate two other PEGs, PEG20k-COOH and PEG1.9k-COOH. The product of each step was confirmed with NMR and FT-IR.

Optimization of conjugation reaction.

With the grafting-to approach, we explored three NHS/EDC catalyzed –NH₂ and –COOH conjugations, 1) to activate protein carboxyl groups and react with PEG-NH₂, 2) to activate carboxylated PEG and react with protein amines, and 3) to mix the protein, carboxylated PEG, and catalysts.^[62] After varying the reaction time, catalyst amount, and feeding ratios (details see SI), we found the third approach optimal (as confirmed by SDS-PAGE and activity tests). We, henceforth, prepared all hybrids using this approach. For simplicity and clarity, we will designate each hybrid based on the PEG m.w. and the feeding ratio. For example, “PEG5k 100:1” denotes a hybrid was prepared with PEG5k using a polymer/protein feeding ratio of 100:1. Note that this nomenclature does not mean 100 polymer chains are virtually attached to each protein. The actual attachment number is much less than the feeding ratio (see below discussions) due to the low reaction efficiency.

Electrophoresis confirms the formation of hybrids.

We conducted SDS-PAGE to estimate the m.w. of the formed hybrids. Prior to the test, each sample was washed with Amicon filtration concentrators (30 kDa) to remove any unreacted protein (18.7 kDa) and polymer. As shown in Figure 1, for all samples but the wildtype protein (Figure 1A), broad bands are observed which indicate heterogeneous m.w. of hybrids. For PEG5k-based hybrids, at 500:1 feeding ratio (Figure 1C), the m.w. ranges from

~50 to >100 kDa. Given a total of 13 lysines in T4L, this feeding ratio seemed sufficient to “saturate” all available amines. The bands consistent with very high m.w. (>80 kDa) may originate from the inter-molecular coiling of two or more hybrids. Similar finding is true at 250:1 ratio. At lower ratios (ca. 100:1 and 150:1; Figures 1E and 1F), the m.w. are less heterogeneous (30–70 kDa), meaning 2–10 PEG5k per protein. The light band intensities are caused by the low yield due to the low feeding ratios (note that unreacted proteins or PEG were removed). For PEG1.9k-based hybrids, at 500:1 feeding ratio, we observed a broad band close to 25–45 kDa (Figure 1B), indicating up to 13 PEG1.9k molecules were attached, consistent with the available lysines per protein. For PEG20k, due to the large size conjugation efficiency was much lower, resulting in less polymer chains per protein. Furthermore, the high viscosity of the polymer limited our attempts to wash off the unreacted protein and polymer. These two factors resulted in a low m.w. (30–40 kDa) band for each feeding ratio (100:1 and 25:1; see Figure S1), indicating on average 1 PEG20k was attached. Due to the complex processes of gel sample preparation, random hybrid dissociation and coiling were present, preventing the use of SDS-PAGE to probe details (such as m.w.) of the hybrids. Nevertheless, the gel indicates the formation of hybrids with heterogeneous sizes.

Global structural insights from AFM.

AFM was conducted on several representative samples to visualize the global structure of the hybrids. The general trend is, upon conjugation with polymers, the particle size of each sample (Figures 2A-2D) is larger than that of the protein alone (Figure 2E). The heights of the largest dots of the images are consistent with this trend (Figure 2F). In addition, the particle size is highly heterogeneous, consistent with the gel. With the same feeding ratio (ca. 100:1), the hybrid size formed by PEG20k and T4L is larger than that of hybrids formed by PEG5k (Figures 2A VS 2C; Figure 2F red VS light blue); with the same PEG, the particle size is larger for hybrids formed with a higher feeding ratio (Figures 2A VS 2B; Figures 2C VS 2D). The hydrodynamic radii of coiled PEG5k and PEG20k in water are known to be ~2.2 and ~5.1 nm, respectively.^[63] Given the size of T4L (3–5 nm), the AFM images suggest ~one layer of PEG docked on the T4L (increasing the size of T4L by twice of the PEG radius). The AFM images confirm the hybrid formation and suggest that PEGs in hybrids are likely in a coiled state. However, it is still unclear if the PEGs rest on the surface of the protein or staying away and if the protein remains in the native structure. These issues will be addressed later.

Hybrids have antibacterial activity.

The *Micrococcus lysodeikticus* cells have been an effective platform to assess the antibacterial function of lysozymes.^[64,65] We, therefore, also employ the same approach^[47,48] to evaluate the activity on a total of 7 hybrids. The general trend follows our expectation: the more PEG attached per T4L, the higher the activity loss; the longer the attached PEG, the higher the activity loss. In detail, for PEG5k, at low feeding ratios (100–150:1; Figure 3), the slopes of the Optical Density at 450 nm (OD_{450nm}) are ~50% of that of the wildtype protein, indicating partial activity loss. At higher feeding ratios (250–500:1; Figure 3), there is almost no activity observed. Similar trends hold for PEG20k, wherein hybrids prepared with a 25:1 ratio yields a higher activity than those of 100:1. At 100:1

ratio, PEG20k shows less activity than PEG5k with the same feeding ratio, indicating the longer chain causes more disruption to the activity. At 500:1, PEG1.9k showed almost no activity, indicating the more the protein amines occupied, the less the activity, even if the polymer chain is relatively short.

CD shows marginal T4L secondary structural change.

To probe the reasons of the activity loss, we went on to understand the structural basis of hybrid function loss,^[62] which required the investigation of both the substrate access pathway and the protein structure. We started with checking the secondary structure of the hybrid via CD spectroscopy. In comparison to the native protein, our CD data shown in Figure 4 indicates only a minor difference between the wildtype and the hybrids. Quantitative analysis of the ellipticity of the CD data indicates ~5–6% loss in helical content, consistent with 1–2 turns of helical structure perturbation.^[66] Additional CD data on other samples (Figure S2) are also in line with this finding. At this point, two questions need to be answered: 1) which helices are disrupted and 2) is such a small structural change enough to cause the activity loss?

EPR reveals regions of protein disrupted by PEG.

To probe the local structural changes using EPR, we created seven cysteine mutants of T4L α -helices (Figure 5A) using site-directed mutagenesis, one at a time. Each mutant was then expressed, purified, and reacted with a methanesulfonylthioate moiety. A stable radical was, therefore, attached to the cysteine via the formation of a disulfide bond. The spin label sidechain is often named as “R1” (more details see SI). Each mutant was then hybridized with the three PEGs, PEG1.9k, PEG5k, and PEG20k at various ratios, one at a time. Representative data on five sites of PEG5k-T4L at four ratios, 100:1, 150:1, 250:1, and 500:1 are shown in Figure 5 (additional data shown in SI; spectra in water and on a solid support are shown for comparison). These data indicate two categories of spectral linewidth changes, a gradual broadening (65R1, 72R1, 89R1, 118R1, and 151R1) and almost no broadening (44R1 and 131R1).

The EPR linewidth is dependent on three factors, the protein rotational tumbling, the conformational dynamics of the helix that is labeled, and the rotational diffusion of the spin label.^[67] While the first and the third factors are often on the order of ns, the motion of structured α -helices is much slower (μ s-ms). We, therefore, ignore this term in our later analysis. In water (the top spectrum of each sub-figure) protein tumbling and spin label motion result in sharp line shapes for all sites due to the motional average. On a solid support such as the CNBr-activated sepharose (the last spectrum of each sub-figure), the protein rotational tumbling is completely restricted.^[68] The resultant spectra reflect the spin label intrinsic motion. In between these two extremes are our hybrids. Once the protein molecular weight is increased due to conjugation with polymers, the hybrid's hydrodynamic radius is likely to be increased. This results in a slower protein rotational tumbling due to the relation of rotational correlation time, τ_c , with the molecular hydrodynamic radius, r :

$$\tau_c = \frac{4\pi\eta r^3}{3kT} \quad \text{Equation 1}$$

where η is the viscosity of the solvent, k is the Boltzmann constant, and T is the temperature. Assuming the label's intrinsic motion is unchanged upon polymer attachment (the label does not make contact with the polymer; reasons see below), a slower protein rotational tumbling (a longer τ_c) often results in a broader spectral line shape, which is what we observed for the 5 out of 7 studied sites (three shown in Figure 5; data on 72R1 and 118R1 shown in Figure S3). Indeed, we found a correlation of the rate parameter of these spectra with the average molecular weight of the hybrids (see below).

To quantitatively assess the broadening caused by PEG conjugation, we performed the half-height peak width measurements for 65R1 (Figure 5B) with different feeding ratios. Our analysis indicated an increase from 3.47 G (no PEG) to 8.88 G (500:1 feeding ratio) in the low-field peak, while a much smaller extent of increase is obtained in the central peak (2.51 G to 3.47 G). Clearly, the broadening effect is the most significant in the low-field region. The same order of peak width increase was observed for other labeled sites (except for 44R1 and 131R1; reasons see below).

The 44R1 and 131R1 (Figures 5E and 5F) did not show the expected line broadening at all feeding ratios. Since we have confirmed the increase in hybrid m.w. by gel and other labeled sites, we speculate that the backbone of these two sites must be disrupted to a less structured conformation, so that more motions contribute to the motional average of spectra. Such contribution is so strong that the increase in m.w. did not broaden the EPR linewidth. Given the high number of protein amines near these two sites (green spheres of Figure 5A), it is reasonable for PEG to disrupt these regions. Such disruption must only occur locally, because the CD data indicated only marginal changes in the overall secondary structure.

Similar findings hold for the other two series of PEGylated hybrids. As shown in Figures S4 and S5, for all but the 44 and 131 sites a gradual spectral broadening is observed. This indicates even the short PEG1.9k can induce structural disturbance once attached. It should be noted that since none of the spectra show “unexpected” broadening, it is less like for the proteins surface (at the labeled sites) to make direct contact with PEG. Otherwise one would observed broadened spectra due to the restrictions of the spin label induced by polymer. This finding suggests that the PEG chains tend to stay away from, instead of wrapping around or directly sitting on, the protein surface. This is a direct experimental evidence showing PEGs tending to stay away from the host protein. In combination with AFM findings, our results suggest that the PEG chain near the attachment point tend to extend away from the protein surface while the rest of the polymer form coiled conformations/shapes.

EPR linewidth is correlated with hybrid molecular weight.

To quantitatively understand the spectral broadening, we performed a 6-step analysis procedure. Step 1, prior to PEGylation, we obtained the time constant from spectral fitting, $\tau_{\text{EPR, -PEG}}$, using a software based on the MOMD model (Figure S8) developed by Prof.

Freed and coworkers.^[69] A brief description of the simulation procedure is provided in the SI. Step 2, prior to PEGylation, we obtained the rotational correlation time, τ_c , of the 18.7 kDa protein, T4L, from NMR data base.^[70] This is ~11.2 ns. Step 3, we calculated the rotational diffusion time constant of the spin label, τ_{SL} , using Equation 2:

$$\frac{1}{\tau_{EPR}} = \sqrt{\frac{1}{\tau_{SL}} \frac{1}{\tau_c}} \quad \text{Equation 2}$$

Note that τ_{SL} is independent of PEGylation. Step 4, upon PEGylation of T4L, we again used the MOMD model to obtain the EPR time constant, $\tau_{EPR, +PEG}$. Note that we use “+PEG” to distinguish the EPR constant from that in step 1. Step 5, by substituting τ_{SL} , from step 3 and $\tau_{EPR, +PEG}$ From step 4, we used Equation 2 again to calculate the τ_c , +PEG. Lastly, we correlated $\tau_{EPR, +PEG}$. With the hydrodynamic radius, r , via Equation 1; the r was further correlated with the m.w. via

$$r \approx \sqrt[3]{\frac{3M}{4\pi\rho N_A}} + r_w \quad \text{Equation 3}$$

where M is the m.w., ρ is the density, and r_w is the hydration radius of the molecules, and N_A is the Avogadro's number. The average protein and PEG density is 1.37 g/cm³ and 1.1–1.2 g/cm³, respectively. Upon formation of the hybrids, the ρ is roughly 1.12–1.2 g/cm³. Taken together, τ_c is roughly correlated with m.w. as $\tau_c \sim \text{m.w.} \cdot 0.7$. Upon taking account of the influence of PEG on the average density, we obtained the m.w..

In detail, we chose 65R1 and 151R1 to perform the analysis because these two sites have single-component spectra (for simplicity) and high signal-to-noise ratios (for precision). The best fit to each data are shown in Figures S9 and S10. The time constant from spectral fitting, τ_{EPR} , is shown in Table S1. Using a τ_c of ~11.2 ns,^[70] for the PEGylated 65R1, without PEGylation, the $\tau_{EPR, -PEG}$ is 1.1 ns (Table S1 row 1). These two values yield a τ_{SL} of 0.108 ns for 65R1. We then followed step 5 to estimate the τ_c , +PEG of the corresponding hybrid (see Table 1). Lastly, the m.w. was calculated for each hybrid based on discussions of step 6 (see above) and shown in Table 1 as well. The uncertainties in the m.w. originate from uncertainties of τ_{EPR} are ± 2.0 kDa. The average m.w. estimated from simulation are shown by the red bars of Figure 1. It has to be noted that the red bars are slightly lower than the mean position of the wide band of each hybrid. This is possibly due to the fact that the low m.w. hybrids contribute sharper spectra and dominate the final EPR linewidth. Nevertheless, a linear correlation of m.w. and τ_{EPR} was found (Figure S11), which can be used to estimate the average m.w. of the synthesized hybrids. EPR linewidth is, therefore, an alternative approach for estimating hybrid size.

AuNPs-hybrid interaction reveals the changes in surface charge.

Our EPR work revealed the protein regions which were disrupted by the attachment of PEG. However, such small perturbation in regions far away from the T4L active site is less likely to be entirely responsible for the activity loss. In fact, at low feeding ratios the disturbance

had occurred (no broadening at these sites) but the hybrid was still partially bioactive. Another possibility is PEG somehow disrupts the interaction of the protein with the substrate, which changes the surface charge of the hybrid and/or blocks the access pathway of substrates. Our recent work showed that T4L was able to trigger the aggregation of small AuNPs (14 nm) due to the positive surface charge.^[47] Therefore, AuNPs are effective sensors to probe T4L charges. As shown in Figure 6, both direct visualization (Figure 6A) and UV-vis spectra (Figure 6B) indicate almost no aggregation was triggered by most of our hybrids, confirming the change in surface charge. The only exception is the PEG20k 25:1, wherein the unwashed native protein may cause the shift (Figure 6C) and aggregation. The neutralized protein surface might also reduce the chance of the often negatively charged substrates (cell walls) interacting with the protein active site, which leads to the activity loss.

Impact of PEG length and abundance on the activity loss.

PEG is also possible to block the substrate access pathway. This is especially true at low feeding ratios. For example, at 100:1, the number of attached PEG5k and PEG20k per T4L is likely to be close, leading to similar change in surface charge. However, the longer the PEG chain, the higher the activity loss (Figure 3). This means that the longer PEG chain has a higher chance to block the substrate access pathway (Figures 7A VS 7C). High PEG abundance leads to complete substrate access blockage (Figures 7B and 7D) and activity loss. Taken together, our findings suggest that the bioactivity loss is related to a combination of three factors, the minor structural disruption at two regions, surface charge change, and PEG chain blocking substrate access. The latter two seem to be more important; low abundance with intermediate chain length seems to be optimal for hybrids' bioactivity.

Broader implications.

Our activity tests (Figure 3) suggest the PEGylated T4L can be great potential antibacterial materials. Herein PEG protects the protein surface from the environment and T4L performs antibacterial functions.^[64,65] Our structural findings provides indications for optimizing the activity. First, PEG is a good candidate for preparing hybrids since it does not disturb the protein surface (but rather stays away). It caused only minor structural disruption to T4L which is not the primary reason for the activity loss. Second, the abundance and length of attached PEG chains should be optimized, so that sufficient protection is retained and minimal substrate blockage and/or protein charge change is caused. Lastly, a potential drawback of our conjugation reaction is the difficulty to control the polymer labeling site(s) and abundance. If possible, it is ideal to control the PEG attachment position, so that the PEG stays far away from the active site. This is one of our future directions.

Using spin density to measure hybrid concentration.

One of the challenges to investigate hybrids is to determine their concentration. The stock concentrations of our hybrids cannot be determined by protein A280 nm due to the absorption of PEG at this wavelength (see Figure S6). We used an EPR-based, external standard calibration method to determine the hybrid concentrations for our activity assay and other measurements. The details of procedure are provided in the SI (Figure S7). The EPR approach is especially helpful when a polymer with a high adsorption near 280 nm was

conjugated to proteins or the unreacted polymers were not completely removed after the conjugation.

Conclusions

We performed a comprehensive study on a series of antibacterial PEGylated proteins. Using a combination of several techniques, we found that three factors are responsible for the bioactivity loss of these hybrids, the minor, local protein structural perturbation, the surface charge change, and the PEG chain blocking substrate access. The latter two were believed to be dominant. Based on this, we proposed future directions to optimize hybrid materials. Our data also suggest that PEG is a good candidate for making hybrid materials since it tends to stay away from the surface of our protein, as suggested by EPR and AFM. Third, we found a rough correlation between EPR linewidth and the average molecular weights of the hybrids, providing an alternative approach to estimate hybrid size. Lastly, we pointed out the possibility of using our hybrid for antibacterial applications wherein PEG helps stabilize the protein from the complex environment. Structural insight of hybrids plays a key role in understanding and refining hybrid design. Our data are of fundamental importance in guiding future hybrid design. We also aim to generate some excitement in developing protein-polymer hybrid materials based on a combination of AFM and EPR structural investigations.

Experimental Section

The mutants of T4L were expressed, purified, and then desalted using previously reported procedure (for details see the Supplementary Information, SI).^[48] The commercial hydroxylated PEGs were purchased from Sigma Aldrich. The details of polymer modification and polymer-protein conjugation are provided in the SI. The AuNPs were prepared using a known procedure with minor modification.^[47] UV spectra were obtained with the NanoDrop UV-vis spectrophotometer (Thermo Scientific ND-2000 C) at the Core Biology Facility of Department of Chemistry and Biochemistry, North Dakota State University (NDSU). CD data were obtained with Jasco J-815 spectropolarimeter at the core facility of Department of Pharmaceutical Sciences, NDSU. The AFM images were acquired under ambient conditions using a commercial atomic force microscope (NT-MDT NTEGRA AFM; details see SI). All CW EPR data were acquired with a Varian E109 and a cavity resonator. The activity assay was conducted as described in the SI.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. SDS-PAGE of the wildtype T4L (A) and a few representative hybrids: PEG1.9k 500:1 (B), PEG5k 500:1 (C), PEG5k 150:1 (D), PEG5k 250:1 (E), and PEG5k 100:1 (F). The red solid or dotted bars are the average m.w. of the hybrid estimated using EPR spectral analysis. Minor bands at ~20 and ~30 kDa in (D) and (F) may be caused by partial dissociation of the hybrids during gel sample preparation (see SI).

Figure 2.

The AFM images of four representative hybrids, PEG20k 100:1 (A), PEG20k 25:1 (B), PEG5k 100:1 (C), and PEG5k 500:1 (D), and the wild type T4L as a control (E). The line profile of the brightest dots in each AFM image: red= A; light red= B; light blue= C; blue= D; black= E.

Figure 3.
Activity assays of the samples involved in this study as color-symbol coded in the set.
Discussions see main text.

Figure 4. The CD data of the hybrids formed by T4L and PEG5k with different feeding ratios. Almost all hybrids show small but noticeable deviations from the wild type protein.

Figure 5. CW EPR spectra of representative T4L mutants in water (top), immobilized on a solid support (bottom), and upon conjugation with PEG5k under different feeding ratios. The vertical dotted lines are to guide the line shape changes.

Figure 6.

(A) Direct visualization of AuNPs color changes upon addition of T4L and hybrids. The time-resolved UV-vis spectra of AuNPs upon addition of two hybrids are shown in (B) and (C). A shift in UV-vis spectra (C) is consistent with the color change observed in (A).

Figure 7.

The structural basis of hybrid activity loss. (A) At low abundance, although local structural disturbance was present, the hybrids are partially active since the substrate access is available. (B) At high abundance the substrate access is blocked, leading to the loss in activity. (C) At low abundance, the longer the PEG chains, the higher chance for the substrate pathway to be blocked. (D) At high abundance, even short PEG chains are sufficient to create substrate blockage, leading to the loss in activity.

Table 1.

The rotational correlation time, τ_c , of two T4L mutants and the associated hybrids determined by analysis based on Equation 2. The corresponding average m.w. was calculated based on τ_c and r (Equation 3).

PEG m.w.	65R1 Feeding Ratio	τ_c (ns)	m.w. (kDa)	151R1 Feeding Ratio	τ_c (ns)	m.w. (kDa)
5k	0	11.2	18.7	0	11.2	18.7
	100:1	20.5	29.4	100:1	20.3	29.0
	150:1	24.3	34.7	150:1	26.7	38.2
	250:1	29.7	42.4	250:1	28.8	41.2
	500:1	44.8	64.0	500:1	44.4	63.4
1.9k	500:1	22.5	32.2	500:1	24.3	34.8
20k	25:1	18.1	25.9	25:1	20	28.6
	100:1	19.7	28.2	100:1	23.4	33.4