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Supporting Information Placeholder

ABSTRACT: Protein-ligand conjugations are usually carried out in aqueous media in order to mimic the environment within which the conjugates will be used. In this work, we focus on the conjugation of amphiphilic variants of elastin-like polypeptide (ELP), short elastin (sEL), to poorly water-soluble compounds like OPPVs (*p*-phenylene vinylene oligomers), triarylamines, and polypyridine-metal complexes. These conjugations are problematic when carried out in aqueous phase because hydrophobic ligands tend to avoid exposure to water, which in turn causes the ligand to self-aggregate and/or interact non-covalently with hydrophobic regions of the amphiphile. Ultimately, this behavior leads to low conjugation efficiency and contamination with strong non-covalent "conjugates". After exploring the solubility of sEL in various organic solvents, we have established an efficient conjugation methodology for obtaining covalent conjugates virtually free of contaminating non-covalent complexes. When conjugating carboxylated ligands to the amphiphile amines, we demonstrate that even when only one amine (the N-terminus) is present, its derivatization is 98% efficient. When conjugating amine moieties to the amphiphile carboxyls (a problematic configuration), protein multimerization is avoided, 98-100% of the protein is conjugated, and the unreacted ligand is recovered in pure form. Our syntheses occur in "one pot" and our purification procedure is a simple workup utilizing a combination of water and organic solvent extractions. This conjugation methodology might provide a solution to problems arising from solubility mismatch of protein and ligand, and it is likely to be widely applied for modification of recombinant amphiphiles used for drug delivery (PEG-antibodies, polymer-enzymes, food proteins), cell adhesion (collagen, hydrophobins), synthesis of nanostructures (peptides) and engineering of biocompatible optoelectronics (biological polymers), to cite a few.

INTRODUCTION

Protein post-translational modification has an important role in nature. Acylation, methylation, phosphorylation, glycosylation and sulfation, among other chemical reactions, are continuously used by biological systems to augment or alter protein function. What nature has discovered and naturally selected through evolution, scientists try to reproduce in the laboratory using available chemical tools to yield conjugates with new critical functions.[1, 2] For example, recombinant antibodies are conjugated to drugs for targeted delivery to tumor cells[3] or pathogens[4], and natural polymers are chemically modified to sense and interact with their environment.[5-7]

Conjugations to biological materials are typically carried out in biologically ambient conditions (that is, aqueous solvent, ≤ 37 ºC, pH 6–8) so as not to disrupt protein architecture and/or function. While some conjugations are straightforward, problems arise when amphiphilic proteins or polymers are conjugated to hydrophobic ligands.[8-10] Hydrophobic ligands by definition reorder water and ultimately avoid exposure to the aqueous phase by aggregating or by seeking hydrophobic regions within the amphiphile.[11-14] As a result, the ligand availability for covalent conjugation is reduced and the conjugation is inefficient. Considering that the interaction between an amphiphile and a hydrophobic ligand can be very strong and that covalent conjugation might

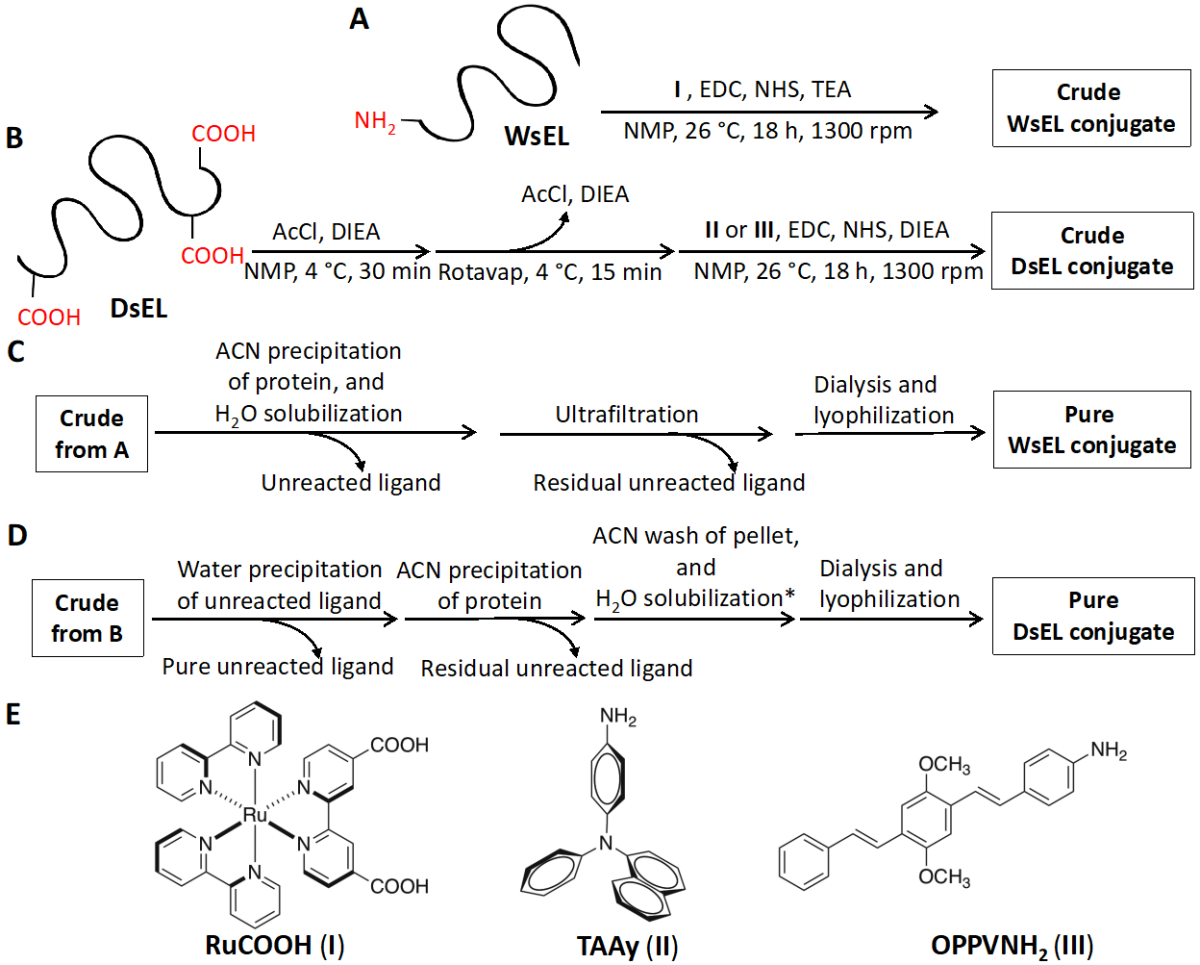

Figure 1. Scheme illustrating amine or carboxyl-mediated functionalization of an amphiphilic protein (short elastin, sEL) with poorly water-soluble ligands in organic solvent. (**A**) Reaction conditions for obtaining the WsEL-RuCOOH conjugate. (**B**) Reaction conditions for obtaining the DsEL-TAAy or DsEL-OPPVNH$_2$ conjugates. (**C**) Procedure for the purification of covalently conjugated WsEL. (**D**) Procedure for the purification of covalently conjugated DsEL. *As an alternative method, the water-solubilized protein from the previous step can be extracted with dichloromethane. (**E**) Ligand structures.

be inefficient, it is not surprising that non-covalent interactions are often favored over covalent conjugation. Indeed, this dominating non-covalent interaction between hydrophobic ligands and amphiphiles is often exploited for encapsulation of hydrophobic drugs within micelles.[14-17] Our group has also experienced difficulty in covalently conjugating amphiphilic polymers to hydrophobic ligands, and recently we have exploited the very strong non-covalent interaction between elastin-like polymer (ELP) and *p* -phenylene vinylene oligomer (OPPV) for generating a composite elastin-like polymer which exhibits both pH and temperature-dependent fluorescence emission.[5] However, for most devices or therapeutics, molecularly-defined and covalent conjugates are the desired target.[18-24] Research aimed to increase the efficiency of covalent conjugation, and prevent/disrupt non-covalent interactions is therefore very relevant. One of the most commonly employed methods to modify proteins is the reaction of solvent-accessible protein amines with carboxyl-containing ligands activated by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)/N-hydroxysulfosuccinimide (NHS) chemistry, in aqueous buffers.[25] Although this method is straightforward for hydrophilic proteins, problems arise when using amphiphilic proteins and poorly water-soluble ligands, due to solubility mismatch of the reagents. An efficient reaction typically requires ten-fold or higher excess of ligand over protein, but when a hydrophobic ligand is used such ratios are difficult to achieve, even with the use of organic co-solvents, unless the concentration of reactants is kept very low. Unfortunately, the low concentration of reactants, together with the tendency of the ligand to non-covalently interact with the protein,[11-14, 26] leads to low conjugation efficiency. Furthermore, conjugation through protein carboxyl groups is seldom used, likely because activated carboxyls might react more readily with free amines within the protein than with the ligand amines. Conjugation of amphiphiles to hydrophobic ligands has been performed in organic solvents to prevent the problems mentioned above.[27-29] However in most of these cases conjugation is an extra step within the solid phase synthesis of the amphiphile, so these methodologies do not transfer to post-expression modification of a large amphiphile or protein (usually obtained recombinantly). Furthermore,

recovery of unreacted ligand (desirable when expensive or custom-made ligands are used) is rarely reported.

Here we present a methodology for conjugation of OPPV,[30-32] triarylamine,[33] and polypyridine-metal (Ru) complexes,[34] to variants of the polymer short elastin (sEL, as exemplar amphiphile), in *N*-methyl-2-pyrrolidone (NMP), through the polymer free amines or carboxyls (Figure 1). sEL is an elastin-like polymer (ELP) and member of a broader class of stimuli responsive ''smart'' polymers that exhibit inverse temperature phase transition behavior in response to changes in their environment.[35-38] The selected ligands are examples of: a) substituted OPPV which upon aggregation and strong dipole–dipole solute–solvent interactions exhibit strong solvatochromism[39] (4-[2-(2,5-Dimethoxy-4-styryl-phenyl)-vinyl]-phenylamine, here called OPPVNH_2, Figure 1E);[40] b) electron-rich triarylamines known to be easily oxidized while forming stable polarons and exhibiting noticeable change of coloration[33] (*N*-(4-Aminophenyl)-N-phenyl-1-naphthalylamine, here called TAAy, Figure 1E); and c) polypyridine-metal complexes with high photoluminescence and well-defined, tunable emission spectra based on metal-to-ligand charge transfer,[41] [Ru(2,2'-bipyridine)-2-(2,2'-bipyridine-4,4'-dicarboxylic acid)]$(PF_6)_2$, here called RuCOOH, Figure 1E; and [Ru(2,2'-bipyridine)-2-(5-amino-1,10-phenanthroline)]$(PF_6)_2$, here called $RuNH_2$, Figure S1).[42] Beyond serving as exemplar hydrophobic ligands to develop our conjugation methodology, the covalent conjugation of these molecules to sEL can generate electrochromic and optoelectronic materials with a wide spectrum of applications.[6, 31]

The simple conjugation methodology presented here allows for facile and efficient derivatization of amphiphile amines or carboxyls, minimization of non-covalent interaction between amphiphile and hydrophobic ligand, minimal protein multimerization, separation of the product from non-covalently bound ligand, and recovery of unreacted ligand. Although we report on the conjugation of sEL variants, our methodology could be used for conjugation of any amphiphilic protein or polymer of interest, including: PEGylated antibodies[43] (some of which are soluble in organic solvents[44-46]); modified organosoluble enzyme conjugates such as POXylated[47, 48] and PEGylated enzymes;[49, 50] a plethora of amphiphilic peptides used as molecular building blocks for nanostructures;[51] food proteins used for drug delivery (e.g. gelatin and casein);[52,53] collagen;[54] and proteins such as hydrophobins (fungal derivatives used for their unique surface-active and self-assembly properties).[55]

RESULTS AND DISCUSSION

Conjugation of poorly water-soluble compounds to amphiphilic proteins in aqueous conditions is difficult. Even when the protein solubility is high, the poor solubility of the ligand restricts the range of usable protein concentrations to low values, since a high ligand to protein ratio is usually required. The sub-optimally low concentration of reactants results in low conjugation efficiencies, even after pH optimization, co-solvent use, and long incubation times. In the case of amphiphilic polymer sEL, or any polymer/protein that undergoes temperature dependent phase changes, an additional hurdle is the need to perform the reaction at low temperature, due to sEL coacervation at room temperature.[7, 36, 56] Thus, for example, aqueous conjugation of terpyridine-metal complexes to ELP carboxyls typically resulted in a conjugation yield of only about 45%.[6] Furthermore, when conjugation is performed in water we have observed that even with the least hydrophobic ligand in our suite ($RuNH_2$, Figure S1), a significant percentage of sEL is non-covalently bound to the ligand (Figure 2). Driven by the expectation that a new strategy for conjugation in organic solvents could solve the issues described above, we investigated the solubility of sEL in several organic solvents.

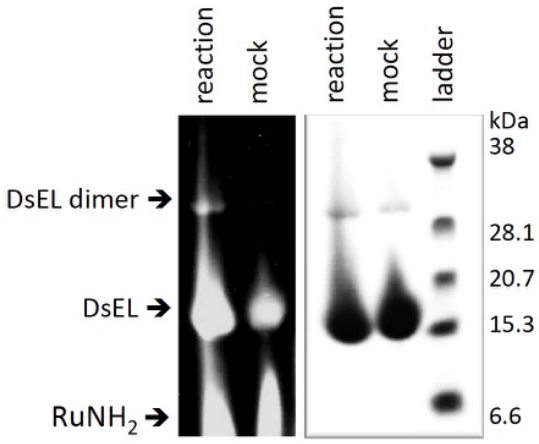

Figure 2. SDS-PAGE shows a very strong ligand-polymer non-covalent interaction when amphiphile-hydrophobic ligand conjugation is performed in water. The reaction of activated (reaction) or non-activated (mock) sEL with $RuNH_2$, in water, was examined by UV transillumination (left, the fluorescence observed is due to the ligand) and by Coomassie blue staining of the protein (right). The higher fluorescence associated with the sEL band in the reaction lane *vs* the sEL band in the mock lane (left side), indicates that there is covalent binding of $RuNH_2$ to the activated sEL. However, the significant amount of fluorescence associated with the sEL band in the mock lane reveals a strong non-covalent interaction between protein and ligand, which withstands the denaturing condition of the gel analysis. The fluorescence at the solvent front reveals a large amount of ligand that could not be removed after multiple ultrafiltration of the conjugation reaction mixture, and that was most likely non-covalently bound to the protein prior to separation on gel.

sEL behavior in organic solvents. The sEL variants used in this work were WsEL, engineered to contain a C-terminal tryptophan (W) for ease of characterization (Figure S2A), and DsEL, containing an extra aspartate (D) upstream from sEL, and (Figure S2B). WsEL contains one primary amine (the N-terminus) available for conjugation to carboxyl-containing ligands. DsEL contains a total of three carboxyls (indicated in red in Figure S2B) available for conjugation to amine-containing

ligands. Both DsEL and WsEL are very soluble (up to 50 mg/mL) in dipolar aprotic solvents dimethylformamide (DMF), dimethylsulfoxide (DMSO) and NMP, but insoluble in acetonitrile and dichloromethane.

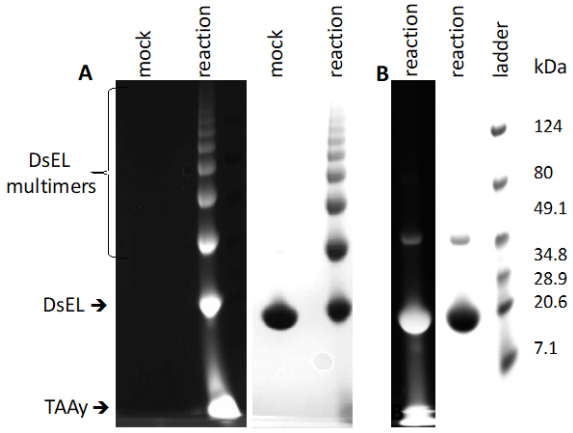

Figure 3. SDS-PAGE analysis shows that activation of protein carboxyls leads to uncontrollable protein multimerization probably due to intermolecular reactions. Reaction between activated (reaction) or non-activated (mock) DsEL and TAAy, were analyzed by either UV transillumination (left panels of A and B) or Coomassie Blue stain (right panels of A and B) of SDS-PAGE gels. Amines in DsEL were either non-protected (**A**) or protected (**B**) by acylation. When protection was not performed, we observed protein multimerization (**A,** reaction lanes). On the contrary, with protection only a miniscule amount of DsEL dimer was observed (**B,** reaction lanes). The product of mock reaction (**A**, mock lanes) appears to be free of non-covalently bound TAAy, as revealed by the absence of visible fluorescence associated with the DsEL band and at the solvent front. However, analysis of the reaction product reveals free TAAy at the solvent front, suggesting an interaction between unreacted and covalently-bound ligand, disrupted during gel electrophoresis.

Among the compatible solvents, we have chosen NMP for the work presented here. We have also observed that sEL does not coacervate at room temperature (as it does in water) in the compatible organic solvents. Notably, even after 24 h exposure of sEL to organic solvent, upon removal of these solvents and resuspension of the protein in water, the reversible temperature-dependent coacervation typical of sEL[7, 36, 56] is still observed (Figure S3).

Conjugation strategies and conjugate purification. WsEL was conjugated to RuCOOH (Figure 1A, mass spectrum of WsEL Figure S4), while DsEL was conjugated to $OPPVNH_2$ or TAAy (Figure 1B, mass spectrum of DsEL Figure S5). Conjugation reactions were performed in NMP, using EDC/NHS activation of carboxyls in the ligand (WsEL conjugation, Figure 1A) or in the protein (DsEL conjugation, Figure 1B). Ten-fold excess of ligand with respect to the protein conjugation sites was typically used, and the reaction was incubated at 26 °C overnight. In our early conjugation attempts, we observed a significant amount of DsEL multimerization (Figure 3A) likely due to intermolecular reaction of activated protein carboxyls with protein amines. Protein multimerization was not observed in WsEL conjugation possibly due to the lower carboxyl to EDC/NHS ratio and to the higher availability of ligand carboxyls (free and more mobile) versus protein carboxyls (protein-bound and possibly buried) for reaction with protein amines. To prevent DsEL multimerization, protein amines were protected by acylation (mass spectrum, Figure S5B). This pretreatment dramatically reduced DsEL multimerization (Figure 3B). The removal of the protecting reagent, acetyl chloride, necessary to avoid acylation of the aminated ligand, was achieved very

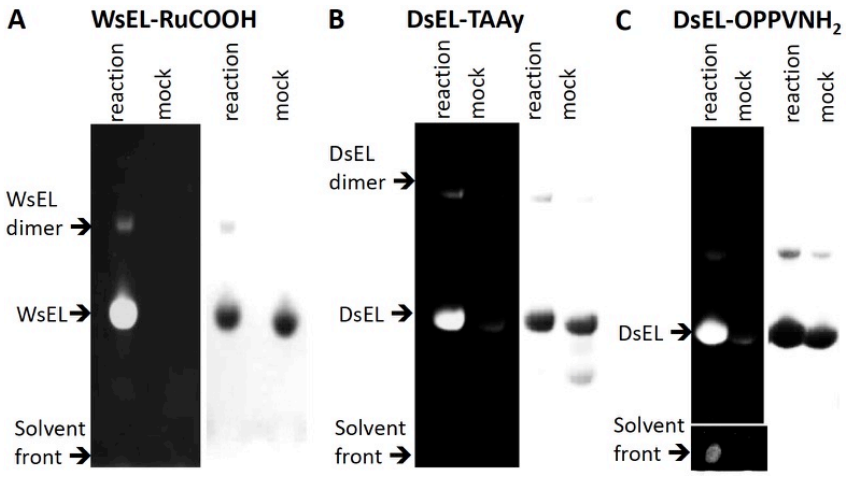

Figure 4. SDS-PAGE analysis shows that our methodology of amphiphile conjugation in organic solvent produces monomeric conjugates virtually free of non-covalently bound ligand. The purified product of reaction between activated (reaction) or non-activated (mock) RuCOOH and WsEL (**A**), and between activated (reaction) or non-activated (mock) DsEL and either TAAy (**B**) or $OPPVNH_2$ (**C**) were analyzed by SDS-PAGE. Gels were examined by UV transillumination (left of each section) and Coomassie Blue staining (right of each section). The fluorescence (derived from the ligand) is mainly associated with sEL (only minimally dimerized) in the reaction lanes. Negligible amount of ligand non-covalently bound to the protein is observed in conjugation to TAAy or $OPPVNH_2$, as shown by the minor amount of fluorescence associated with the DsEL band in the mock lanes. Trace amount of ligand non-covalently bound to covalently-bound ligand (migrating with the front of the solvent in denaturing conditions) was observed for DsEL-$OPPVNH_2$ by UV transillumination captured with long exposure time (**C**).

conveniently by brief rotatory evaporation at low temperature (second step in Figure 1B). This step did not result in any significant removal of NMP (a low volatility solvent), and it allowed proceeding to the next reaction step within the same vessel. To monitor non-covalent conjugation, mock reactions free of EDC and NHS (i.e. containing non-activated protein or ligand) were always run in parallel with the conjugation reactions. At the end of the reaction, addition of water caused precipitation of most unreacted ligand in DsEL conjugations (Figure 1D). Extraction of the conjugated protein by precipitation in acetonitrile (ACN) helped remove more unreacted ligand in DsEL conjugation reactions (Figure 1D, second step) and was used as an initial purification step for WsEL conjugation (Figure 1C, first step). Residual unreacted ligand in DsEL conjugation reactions was removed by either acetonitrile wash of the protein pellet (Figure 1D, third step) or by dichloromethane extraction of solution obtained by resuspending the protein pellet in water (Figure S6). For WsEL conjugation, residual unreacted ligand was removed by ultrafiltration of the water-solubilized protein pellet obtained by ACN precipitation (Figure 1C, second step). After the work up procedure, conjugated sEL retained the thermal responsiveness of the unmodified sEL. Purified WsEL-RuCOOH was completely free of non-covalently bound ligand, as shown by UV transillumination of SDS-PAGE gels (no fluorescence associated with the protein band in the mock reaction nor detected at the solvent front, Figure 4A) and by the UV/Vis analysis (no ligand absorbance detected in the mock reaction). A minuscule amount of ligand non-covalently bound to the protein was observed in purified DsEL-TAAy and DsEL-$OPPVNH_2$, as shown by UV transillumination of SDS-PAGE gels (minor amount of fluorescence associated with the protein band was observed in the mock reactions, Figure 4B and C) and by the UV/Vis analysis (modest ligand absorbance in mock reactions, Figure S7B and C). In the case of DsEL-$OPPVNH_2$ negligible amount of ligand non-covalently interacting with the covalently-bound $OPPVNH_2$ was detected by SDS-PAGE with UV transillumination at long exposure time (minor amount of fluorescence was observed at the solvent front in the reaction, Figure 4C, see further discussion below).

Conjugate characterization. Successful chemical conjugation of RuCOOH to WsEL was confirmed by Electrospray Ionization Mass Spectrometry (ESI-MS) and NMR (TOCSY and ROESY). ESI-MS revealed a 11,911 Da (m/z) molecule (Table S1 and Figure S4B) consistent with the WsEL-RuCOOH conjugate. Furthermore, the de-convolved spectrum obtained for WsEL-RuCOOH showed no trace of unbound WsEL. Since we have observed that the unconjugated WsEL ionizes better than the conjugated counterpart, this result suggested high protein derivatization. TOCSY and ROESY NMR analysis of free and conjugated WsEL further proved covalent conjugation (Figures 5, S8, and S9). Upon conjugation, the terminal amine of Ala1 becomes an amide and shifts in the spectrum from 8.37 (Figure S8A), to 9.53 (Figure S8C) ppm while the Hα proton of Ala1 shifts from 4.26 to 4.54 ppm (Figure 5) unambiguously indicating ligand coupling. This is further confirmed by ROESY spectra showing cross-peaks between Ala1 NH resonance and resonances of the ligand (Fig. S9).

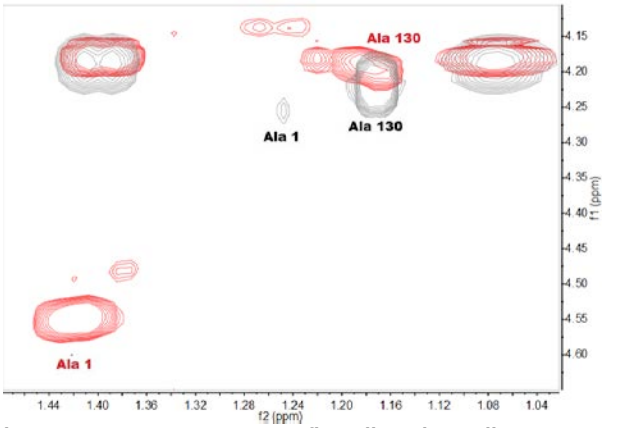

Figure 5. TOCSY spectrum confirms ligand coupling to WsEL Ala1. Upon conjugation of WsEL to RuCOOH the Hα proton of alanine at position 1 (Ala1) shifts from 4.26 to 4.54. Gray: WsEL; Red: WsEL-RuCOOH.

Conjugation efficiency was quantified by UV/Vis spectrophotometry analysis (Figure S7A), which was performed on the other two conjugates as well (Figure S7B and C). Known concentrations of free ligand were used to determine the equation describing the linear correlation between absorbance and concentration (Beer-Lambert law). This equation was then used to convert the ligand absorbance of the purified protein obtained from

Table 1. Efficiency of protein conjugation

Conjugate (protein-ligand)	[Protein] (mM)[a]	[Ligand] (mM)[b]	[non-covalently bound ligand] (mM)	Conjugation efficiency (% protein derivatization)[e]
WsEL-RuCOOH	39.7	38.8	None detected	97.7
DsEL-TAAy	12.1	12.7	0.974[c]	96.9
DsEL-$OPPVNH_2$	58.7	73.7	9.41[c] + 5.81[d]	99.7

[a] concentration of protein from conjugation reaction determined by weight after extensive dialysis and lyophilization
[b] ligand associated with protein from conjugation reaction, as determined by UV/Vis spectrophotometry (red in Figure S7)
[c] ligand interacting non-covalently with the protein from conjugation mock, as determined by UV/Vis spectrophotometry (blue in Figure S7)
[d] ligand interacting non-covalently with the covalently-bound ligand in conjugation reaction, as determined by spot densitometry (Figure S13)
[e] monosubstitution

conjugation reaction or mock (extensively dialyzed, lyophilized and carefully weighed) to concentration or protein-associated ligand. In the case of WsEL-RuCOOH conjugation, there was no detectable non-covalently bound ligand, as demonstrated by the absence of ligand absorbance in the purified protein from mock conjugation, and by SDS-PAGE analysis of mock and reaction (Figure 4A).The percentage of conjugation was calculated using the ratio of the net ligand concentration in the protein obtained from conjugation reaction (i.e. [Ligand] minus [non-covalently bound ligand] in Table 1) to protein concentration ([Protein] in Table 1; obtained by weight). Based on this method, derivatization of the only amine (*N*-terminal) in WsEL was 97.7% complete. We confirmed the conjugation quantification using 700 MHz ^{1}H NMR analysis of conjugated and free WsEL (Figures 6 and S10). Resonance assignments were obtained from TOCSY spectra (Figure S8). To calculate the average ratio of elastin to ligand we used the intensities of individual resonances of Trp132 and the ligand in the aromatic region of the WsEL-RuCOOH spectrum (blue trace in Figure 6), which are well resolved and quantifiable. The resulting estimate of conjugation efficiency was 95 ± 6%. This result corroborates the UV/Vis quantification. The good agreement of the conjugation efficiencies obtained through NMR and UV/Vis analyses renders the two methods interchangeable. Both methods allow the reuse of the analyzed sample, however the UV/Vis method is preferable when dealing with low scale reactions. The conjugation efficiency obtained with WsEL is remarkable, since in other reported attempts to conjugate ELPs through free amines, at least one lysine was inserted in the sequence,[57-62] indicating that the presence of the *N* -terminal amine alone did not afford the desired level of conjugation. Furthermore, when reactions were performed in aqueous phase, even after insertion of extra amines, the ratio ligand:elastin ranged from 0.5 to 1, with 25 to 50% non-site selective amine derivatization.[57, 60] Thanks to the high efficiency of our conjugation method, only one amine is needed in the ELP to obtain near complete derivatization of the polymer, also resulting in knowing the exact location of the derivatization.

Successful chemical conjugation of TAAy and $OPPVNH_2$ to DsEL was confirmed by Matrix Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS, Figure S11) and ESI-MS (Figure S12), respectively (Table S1). The concentration of DsEL covalently linked to TAAy was calculated as described for WsEL-RuCOOH (Figure S7B), except that in this reaction a minuscule amount of non-covalently bound ligand was detected in the mock reaction (Figure 4B, mock lanes and Figure S7B). This ligand was quantified by UV/Vis analysis and used to correct the calculation. The resulting efficiency of conjugation (as % of monosubstituted protein) was found to be 96.9% (Table 1). DsEL-$OPPVNH_2$ also included a small amount of non-covalently linked $OPPVNH_2$ strongly associated to DsEL (Figure 4C, mock lanes). This excess ligand was quantified by UV/Vis analysis of the mock reaction and used for correcting the conjugation efficiency as for DsEL-TAAy (Figure S7C). Another fraction of the non-covalently linked $OPPVNH_2$ (separated from the conjugated protein in denaturing conditions and migrating with the solvent front), seemed to be only associated to the covalently conjugated $OPPVNH_2$ (Figure 4C, left panel, reaction lane, and Figure S13A) and could not be detected by the UV/Vis analysis of the mock reaction. The concentration of this ligand was therefore quantified by spot intensity (Figure S13B) and used to further correct the

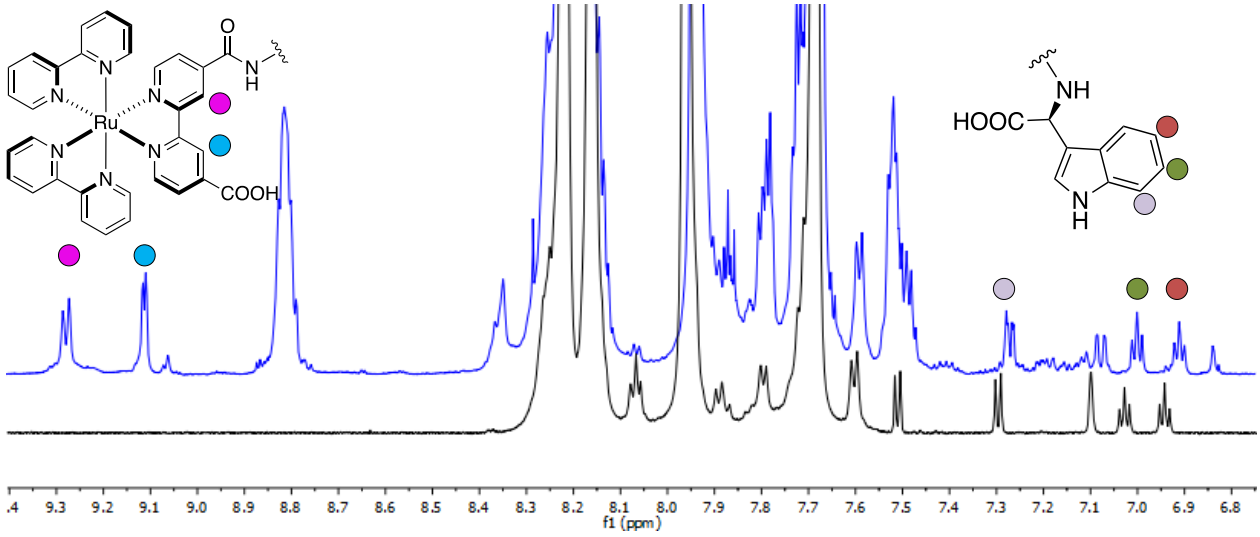

Figure 6. NMR data reveal the conjugation efficiency to be 95 ± 6%. ^{1}H NMR spectra (700 MHz, aromatic region) of free WsEL (black) and WsEL-RuCOOH conjugate (blue) were used to determine conjugation efficiency. The individual Trp132 indole ring resonances and ligand resonances used for integration are labeled with colored dots also used to indicate the correspondent hydrogens in the structure of ligand (RuCOOH, on the left) and Tryptophan (on the right).

concentration of covalently bound $OPPVNH_2$ calculated by the UV/Vis method. The final efficiency of conjugation was 99.7% (Table 1) with virtually no multimerization. These results are noteworthy, since conjugation of amine-functionalized ligand to protein carboxyls with might be problematic Activated protein carboxyls tend to react with protein amines making protein conjugation sites less available for reaction with ligand and causing protein multimerization. In rare reports of the successful use of this conjugation configuration,[63, 64] the protein free amines were protected by lengthy procedures, and extensive dialysis was performed in between protection and conjugation. An additional report states the failure to conjugate amine-containing ligand to protein carboxyls, which prompted the thiolation of the amine-containing ligand, and attachment of maleimide to the protein amines, leading to conjugation through thiol–maleimide Michael addition.[65] Compared to these methods, our procedure, which includes amine protection, carboxyl activation and conjugation to amine moieties in "one pot", is much nimbler and more convenient. The availability of a viable alternative to the most common ligand conjugation through protein amines is very useful when trying to preserve protein activity upon conjugation. For example, an antibody might lose its immunoreactivity upon ligand conjugation through amines, but if the antibody binding site lacks (or includes less critical) carboxyl groups, carboxyl-directed conjugation of an amine-functionalized ligand is less likely to disturb the antibody-antigen interaction.

We verified the preservation of the characteristic ELP reversible phase transition behavior in water following our conjugation methodology. Investigators studying proteins that may denature in organic solvents should verify that the structure and/or function of the derivatized protein is comparable to the structure and function of the unmodified protein.

Recovery of unreacted ligand. Recovery of unreacted ligand might be desirable when custom-made, rather than inexpensive, commercially available compounds, are used. When amine-containing ligands TAAy and $OPPVNH_2$ were reacted with the activated protein carboxyls of DsEL, there was no ligand modification prior to conjugation to protein and therefore the unreacted ligand could be recovered intact. Exploiting the low solubility of ligands in water and the insolubility of sEL in acetonitrile, we were able to separate unreacted ligand from the protein conjugate. Except for residual NMP, the recovered TAAy and $OPPVNH_2$ were fairly pure as determined by ^1H NMR (Figure S14).

CONCLUSION

We have developed and validated a versatile protocol for derivatization of amines or carboxyls within amphiphilic proteins/polymers, with hydrophobic ligands containing carboxyls or amines respectively. Conjugation is performed in organic solvent and purified conjugates are virtually free of non-covalent protein-ligand complexes, which are prevalent when reactions are performed in water. Amine derivatization is quantitative. Carboxyl derivatization, although not quantitative, results in 98% protein derivatization when three carboxyls are present. Furthermore, protein multimerization is avoided and unreacted ligand can be recovered efficiently in high purity.

EXPERIMENTAL PROCEDURES

sEL production. The WsEL expression construct was described previously.[7] The DsEL gene was designed based on the ELP-1 construct described previously.[5-7] Addition of a carboxyl group to facilitate conjugation was achieved by PCR insertion of DNA encoding the N-terminal sequence AGDGS (red in Figure S15). Purified PCR product was subcloned into the BsshII and NheI sites of the POE expression vector (Qiagen). ELP expression was achieved using a protocol adapted from Hassouneh *et al.*[66] and previously described.[5-7] Briefly, plasmids encoding WsEL or DsEL were transformed into competent BL21(DE3) *E. coli* (NEB). Transformed cells were plated on selective solid medium (2xYT + agar + carbenicillin) overnight. A single colony was transferred in 15 mL starter culture and grown at 37 ºC at 300 rpm. Upon becoming visibly cloudy (2-3 h), this culture was used to inoculate 1 L 2xYT. The culture was allowed to grow for 24 h at 37 ºC without induction. Cells were harvested by centrifugation and the periplasmic fraction was obtained by cold osmotic shock in 20% sucrose buffer. sELs were purified by inverse temperature cycling. Protein purity was assessed by SDS-PAGE (Invitrogen, NP0343BOX), and electrospray ionization (Synapt G2 ESI-MS, Waters, Figure S4A and Figure S5A). SDS-PAGE gels were stained using the Coomassie-based stain GelCode Blue (Pierce). Following purification, sELs were dialyzed twice against 4 L of cold deionized (DI) water (first time for 4 h, and second time for 12 h in fresh water) at 4 ºC in a 10 KDa MWCO Slide-A-Lyzer dialysis cassette (Pierce, 66383). Dialyzed proteins were lyophilized and stored dry at -20 ºC.

RuCOOH and $RuNH_2$ synthesis. The ruthenium complexes [Ru(2,2'-bipyridine)-2-(2,2'-bipyridine-4,4'-dicarboxylic acid)]$(PF_6)_2$, RuCOOH, and [Ru(2,2'-bipyridine)-2-(5-amino-1,10-phenanthroline)]$(PF_6)_2$, $RuNH_2$ were prepared and characterized via well-established protocols.[41, 42]

TAAy synthesis. *N*-(4-Aminophenyl)- *N*-phenyl-1-naphthalylamine (TAAy)[39] was synthesized by Pd/C-catalyzed hydrazine reduction of the dinitro compound, which was prepared through the NaH-mediated nucleophilic aromatic substitution reaction of *N*-phenyl-1-naphthalylamine with 4-fluoronitrobenzene.

$OPPVNH_2$ synthesis. 4-[2-(2,5-Dimethoxy-4-styryl-phenyl)-vinyl]-phenylamine ($OPPVNH_2$) was

synthesized by stannous chloride-assisted reduction of the nitro-compound, which was achieved *via* the Horner-Wadsworth-Emmons method by reacting phosphonate carbanions of [4-(diethoxy-phosphorylmethyl)-2,5-dimethoxybenzyl]-phosphonic acid diethyl ester with subsequent benzaldehyde and then 4-nitrobenzaldehyde.[5, 40]

WsEL conjugation with RuCOOH. The required amount of WsEL protein, RuCOOH ligand, EDC, (Thermo Scientific 22980), NHS, (Thermo Scientific 24510) and triethylamine (TEA, Alpha Aesar 12391) were weighed ahead of time into separate small glass vials before combining. 340 μL of NMP (Sigma-Aldrich, 328634) was used to solubilize 6.8 mg of WsEL protein (0.6 μmol). The solution was added to 10-fold molar excess of RuCOOH with respect to WsEL (3.9 mg, 6 μmol). Once RuCOOH was fully solubilized within the protein solution, 10-fold molar excess of EDC and NHS with respect to WsEL (1.2 mg and 1.3 mg respectively, or 6 μmol of each) was added. Finally, TEA (a base used to facilitate ligand COOH activation) was added to the solution in 1.5-fold molar excess with respect to WsEL (12.5 μL of 72 mM TEA stock). The solution was sealed and incubated overnight while mixing (1300 rpm, TAITEC BioShaker N-BR-022UP). The mock reaction was prepared in a similar fashion, except that no EDC nor NHS was present. As a preliminary purification step, acetonitrile (ACN, Fisher Scientific, A955-212), was added to the reaction solution (~4.5:1 volume ratio ACN:reaction). The solution mixture was centrifuged and the supernatant was removed. Since RuCOOH, EDC, and NHS are soluble in ACN, but the protein polymer is not, this step allowed to separate the protein (in the precipitate) from the unreacted reagents (in the supernatant). Sterile-filtered cold water (4 ºC) was added to the precipitate to re-solubilize the conjugated protein. The cold solution was then transferred to a prewashed and cooled 15 mL 10 kDa MWCO centrifugal filter (EMD Millipore) and cold water was added to further dilute the solution. The solution was mixed by pipetting up and down before centrifugation at ~15K rpm, at 4 ºC. The centrifugation progression was checked periodically (every ten minutes) to avoid extensive concentration on top of the filter, thus leading to sticking of conjugated product to centrifuge filter membranes and loss of product. Sequential addition of 4 ºC cold water to the centrifuge filter, re-mixing, and centrifuging was carried out until the flow through was colorless. The conjugated product was collected and dialyzed twice against 4 L of cold DI water (first time for 4 h, and second time for 12 h in fresh water) at 4 ºC in a 10 KDa MWCO Slide-A-Lyzer dialysis cassette. Finally, the product was collected and lyophilized overnight (Labcono, Freezone). Conjugated protein was stored dry at -20 ºC until needed and it was characterized by SDS-PAGE, ESI-MS, UV/Vis and NMR.

DsEL conjugation to OPPVNH$_2$ or TAAy. Conjugation of OPPVNH$_2$ or TAAy with ELP was performed by EDC/NHS coupling, preceded by acetyl chloride (AcCl, Sigma-Aldrich, 00990) protection in the presence of diisopropylethylamine (DIEA, Sigma-Aldrich, 03440) (Figure 1B). A standard reaction utilized 1 to 2 mg of DsEL. Typically, 40 μL of a 2.25 mM solution of DsEL in NMP was mixed with 80 μL of a 5.6 mM solution of Diisopropylethylamine (DIEA) and AcCl in NMP (5-fold excess of DsEL amines). The solution was incubated on ice for 1 h and 45 min. AcCl, and some DIEA, were aspirated by either rotavaping on ice for 10 min or placing the frozen solution in a lyophilizer for 5 min. The acetylation product was characterized by ESI-MS (Figure S5B). Removal of AcCl was necessary to avoid acetylation of the ligand added in the subsequent step. Half of the protected protein (60 μL) was removed and mixed with 60 μL NMP (mock reaction). The remaining 60 μL of the protected protein was mixed with 60 μL of a 22.5 mM EDC and 16.8 mM NHS solution in NMP (reaction, 10-fold EDC and 7.5-fold NHS in excess of DsEL carboxyls). Immediately after this addition, mock and reaction solutions both received 2 μL of a 675 mM solution of OPPVNH$_2$ or TAAy in NMP (10-fold in excess of DsEL carboxyls) and 2 μL of a 13.5 mM DIEA solution in NMP (to facilitate activation of DsEL carboxyls in the reaction solution). Both solutions were incubated at 26 °C for 12 h, with shaking (1300 rpm). A small fraction of reaction or mock solution (2 μL) was mixed with PBS (10 μL), causing the excess unreacted OPPVNH$_2$ or TAAy to precipitate. This step was needed to avoid excessive fluorescence coming from the unreacted ligand, and clear visualization of the fluorescence associated with DsEL, in the subsequent SDS-PAGE analysis. Upon centrifugation (15,000 rpm, 10 min, 4 °C), supernatants were mixed 1:1 with loading buffer, and analyzed by SDS-PAGE. This analysis was needed to verify covalent conjugation in the reaction and lack of covalent conjugation in the mock before subsequent workup. Unreacted ligand was precipitated from the remainder of reaction or mock solutions by adding 5 volumes of cold water and then centrifuging the solution. This step allows for recovery of pure unreacted OPPVNH$_2$ or TAAy (in the precipitate). Supernatants were frozen and concentrated in a lyophilizer for roughly 15 min (wherein the volume was reduced from 120 μL to 40 μL). DsEL was precipitated by addition of 40 μL ACN. In order to remove residual non-covalently bound ligand, the precipitant was either washed 3 times with ACN, or resuspended in water and extracted 3 times with the same volume of CH_2Cl_2 (VWR, UN 1593). Washed precipitate (if using ACN wash), or protein film between phases (if using CH_2Cl_2 extraction), was resuspended in water and dialyzed twice against 4 L of cold water (first time for 4 h, second time for 12 h in fresh water) in a 10,000 MWCO Slide-A-Lyzer. The dialyzed and purified product of mocks and reactions

were lyophilized overnight and a cottony solid was obtained. Conjugates were stored dry at -20 ºC and characterized by SDS-PAGE, ESI-MS or MALDI-TOF MS, and UV/Vis.

UV/Vis spectrophotometry analysis. The amount of lyophilized protein (reaction or mock) needed to obtain 200 μL solution in double digit micromolar range was weighed and dissolved in DMSO. The absorbance maximum (at 461, 308 and 390 nm for WsEL-RuCOOH, DsEL-TAAy, and DsEL-OPPVNH$_2$, respectively) was measured and recorded. A set of solutions of known concentration of ligand spanning from single to double-digit micromolar concentration were analyzed by UV/Vis, and their absorbance maxima were plotted *vs* the corresponding concentrations. The equation defining the linear correlation between absorbance and concentration (Beer-Lambert law) was used to determine the concentration of ligand present in the protein solution (reaction or mock), using the measured absorbance (A) and the following equation: [ligand] = (A-y axis intercept)/ε (extinction coefficient and slope of the line). For WsEL conjugation, there was no ligand absorption in the mock reaction, therefore ligand concentration in the reaction was actually concentration of covalently-bound ligand. A correction was needed for DsEL-TAAy and DsEL-OPPVNH$_2$. In the case of DsEL-TAAy the concentration of covalently bound TAAy was calculated using the following equation [ligand] = (A reaction-A mock)/ε. The same calculation was used for DsEL-OPPVNH$_2$ but the resulting concentration was further corrected using the spot intensity quantification described next.

Spot intensity quantification of non-covalently bound ligand. SDS-PAGE analysis of purified DsEL-OPPVNH$_2$ reaction revealed a small amount of OPPVNH$_2$ interacting non-covalently with the covalently bound ligand (see buffer front at the bottom of Figure 4C, "reaction lane"). In order to quantify this OPPVNH$_2$ a series of standard OPPVNH$_2$ solutions of known concentration were run on the same gel (Figure S13A). The intensity of standard OPPVNH$_2$ spots was determined by using GeneTools analysis software (Syngene) and plotted against the corresponding concentrations to obtain a calibration curve. The concentration associated with the spot intensity of OPPVNH$_2$ freed from the covalently conjugated protein was determined using the following equation [ligand] = (SI-y axis intercept)/ε, where SI is the intensity of the spot corresponding to the freed OPPVNH$_2$ (Figure S13B). This piece of data was used to further correct the conjugation quantification obtained by UV/Vis spectrophotometry analysis.

Electrospray Ionization Mass Spectrometry (ESI-MS) analysis. A continuous spray ESI-MS configuration was used to analyze a 2 ng/μL conjugate solution in 1:1 H$_2$O:ACN, 1% formic acid (for WsEL-RuCOOH) or in 50 mM ammonium acetate pH 7.0 (for DsEL-OPPVNH$_2$). Measurement was performed on a Waters Instrument (Synapt G2), using MassLynx software. For WsEL-RuCOOH and WsEL the instrument settings were: Capillary kV = 5; Sampling Cone = 25; Extraction cone = 1.2; Source T = 80 °C; Desolvation T = 200 °C; Cone gas flow = 32 (L/h); Desolvation gas flow = 500 (L/h). For DsEL the settings were the same as above, except that the Cone gas and the Desolvation gas flow rates were 200 and 700 L/h respectively. For DsEL-OPPVNH$_2$ and protected DsEL the instrument settings were: Capillary kV = 5; Sampling Cone = 40; Extraction cone = 0.3; Source T = 80 °C; Desolvation T = 100 °C; Cone gas = 97 (L/h); Desolvation gas flow = 648 (L/h). Data were processed using the MaxEnt1 tool of MassLynx. All measurements were conducted using a freshly cleaned cone.

Matrix Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) analysis. Samples were mixed in a 10:1 ratio (matrix:sample) with 10 mg/mL of ultrapure 2,5-Dihydroxybenzoic acid (DHB) (Protea, Morgantown, West Virginia). Prior to sample analysis, the mass spectrometer was externally calibrated with a TOF/TOF Calibration peptide mixture of des-Arg-Bradykinin (1.0 pmol/μL), Angiotensin I (2.0 pmol/μL, Glu-Fibrinopeptide B (1.3 pmol/μL), and adrenocorticotropic hormone (ACTH), (1-17 clip-2.0 pmol/μL), (18-39 clip-1.5pmol/μL), (7-38 clip-3.0 pmol/μL). The 4800 Plus MALDI TOF/TOF Analyzer (ABSciex, Foster City, CA, USA) was used in positive reflector mode. Data were collected in manual mode and utilizing random sampling over the entire sample spot. The mass spectrometer is equipped with a 200-Hz frequency Nd:YAG laser, operating at a wavelength of 355 nm. Twenty five sub-spectra for each of 100 randomized positions within the spot (2,500 spectra/spot) were collected in MS-TOF mode and presented as one main spectrum. Fixed laser intensity in MS-TOF reflector positive mode, 3600 (arbitrary units), final detector voltage set at 1.883 KV. Spectral Mass range 1000-2500 m/z, focus mass 1800 m/z. Data were exported to Data Explorer™ Software, Version 4.9 (ABSciex, Foster City, CA, USA) and interpreted using the centroid function of the software.

NMR analysis. All spectra of WsEL and WsEL-RuCOOH conjugate were obtained on a Bruker Avance III NMR spectrometer operating at ^{1}H frequency of 700.13 MHz in DMSO-d$_6$. One dimensional spectra used for conjugation efficiency quantification were obtained using recycle delay of 12 s (D1=10s, AQ=2s) to allow for complete magnetization recovery between scans. 512 scans per sample were collected, resulting in acquisition time of 1.7 h per spectrum. Spectra were apodized with 1Hz exponential function, Fourier transformed and baseline corrected using a polynomial function. ^{1}H-^{1}H TOCSY spectra were obtained using DIPSI2 mixing sequence[67] for spin-lock (40 and 60 ms mixing time), with 2048 data points in t2 and 512 points in t1 and relaxation delay of 3 s. Quadrature detection in

t1 was achieved using States-TPPI scheme. Data were apodized using a cos^2 function, zero filled to a 2048x1024 matrix and Fourier transformed. ^1H-^1H ROESY spectra were obtained using a standard pulse sequence with cw spin-lock[68], with mixing time of 300 or 400 ms. 2048 data points were collected in t2 and 512 points in t1 and relaxation delay was set to 3 s. Quadrature detection in t1 was achieved using States-TPPI scheme. Data were apodized using a cos^2 function, zero filled to a 2048x1024 matrix and Fourier transformed.

ASSOCIATED CONTENT

Supporting Information.

Structure of $RuNH_2$; sEL variants sequences; sEL behavior in organic solvents and water; SDS-PAGE analysis of reaction workup; Calculation of conjugation efficiency by UV/Vis absorbance; TOCSY spectra of WsEL and WsEL-RuCOOH; ROESY spectrum of conjugated WsEL; ^1H NMR spectra of free and conjugated WsEL; Quantification of non-covalently bound ligand by SDS-PAGE and spot intensity; ^1H-NMR of recovered ligands; Primer for DsEL cloning; Table of calculated and found masses.

The Supporting Information is available free of charge on the ACS Publications website (PDF).

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Notes

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ABBREVIATIONS

2xYT, 2x yeast extract tryptone; AcCl, acetyl chloride; D, Aspartate; CH_2Cl_2, Dichloromethane; DI, deionized; DIEA, diisopropylethylamine; DMF, Dimethylformamide; DMSO, Dimethyl sulfoxide; DsEL, short elastin with extra Aspartate (D); EDC, 1-ethyl-3-(-3-dimethylaminopropyl) carbodiimide; ELP, elastin-like polypeptide; ESI-MS, ElectroSpray Ionization Mass Spectrometry; MALDI, Matrix Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry; NHS, N-hydroxysuccinimide; NMP, N-Methyl-2-pyrrolidone; NMR, nuclear magnetic resonance; OPPV, p-phenylene vinylene oligomers; PBS, phosphate buffer saline; sEL, short elastin; SDS-PAGE, sodium dodecyl sulfate poly acrylamide gel electrophoresis; TAAy, triarylamine; TEA, triethylamine; W, Tryptophan (W); WsEL, short elastin with C-terminal Tryptophan (W).

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