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Non-ionic water-soluble "clickable" α-helical polypeptides: synthesis, characterization and side chain modification

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Jinbao Cao[a], Ping Hu[b], Lu Lu[a], Brandon Chan[a], Bing-Hao Luo[b] and Donghui Zhang*[a]

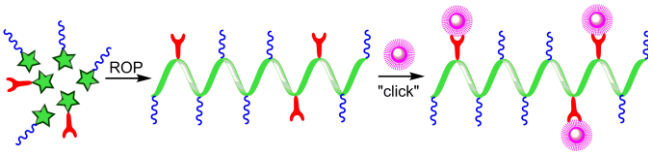
A series of water-soluble non-ionic "clickable" polypeptides has been synthesized by organo-initiated ring-opening co-polymerization (ROP) of γ-propargyl L-glutamic acid N-carboxyanhydride (PLG NCA) and N-ε-2-[2-(2-Methoxyethoxy)ethoxy]acetyl-L-lysine *N*-carboxyanhydride (EG_2-LYS NCA). The pendant alkyne side groups can be modified with azido-containing hydrophobic and hydrophilic bioactive moieties, producing polypeptide conjugates with good water solubility. Circular dichroism (CD) reveals that both the parent polypeptides and the modified polypeptide conjugates maintain high levels of α-helical conformations in aqueous solutions. Preliminary cell study indicated the cell binding peptide GRGDS (Gly-Arg-Gly-Asp-Ser) modified copolymers are able to induce integrin-mediated cell adhesion.

Polypeptides are *α*-amino acid based polymers capable of adopting basic secondary structures such as *α*-helix or *β*-sheets reminiscent of those observed for proteins.[1, 2] Recent development in the controlled ROP has enabled access to well-defined polypeptides with tailorable composition, architecture, functionality and size.[3] In addition, polypeptides are proteolytically degradable and exhibit low cytotoxicity. As a result, synthetic polypeptides have been increasingly investigated as a platform for biomedical applications such as gene therapy[2, 4, 5], drug delivery[6-9] and tissue engineering scaffolds.[10, 11]

The solubility and conformation of polypeptides are strongly dependent on the side chain structures. Water-soluble polypeptides often bear charges on the side chains (*e.g.*, poly(L-glutamic acid), polysulfonium based on poly(L-homocystine)[12]). The electrostatic repulsive interactions between the charges on the side chains cause the polymer backbones to adopt random coil conformations. Moving the charges further away from the backbone has successfully yielded water-soluble ionic polypeptides adopting helical conformations.[13] In spite of their high water solubility, ionic water-soluble polypeptides (*e.g.*, poly(L-lysine)) tend to bind to oppositely charged biomolecules *in vivo*, resulting in cytotoxicity.[14] As a result, non-ionic water-soluble polypeptides are highly sought after to complement the charged polymers for certain biomedical applications (*e.g.*, delivery carrier for hydrophobic therapeutics).

Several non-ionic water-soluble polypeptides that adopt stable secondary structures have been reported. They are synthesized either through post-polymerization modification of polypeptides bearing reactive side chains with appropriate hydrophilic moieties such as monosaccharides and PEG[15-24] or by direct ROP of discrete *N*-carboxyanhydride (NCA) monomers bearing hydrophilic side groups (*e.g.*, glycosylated-L-lysine NCA).[1, 12, 25-28] Most of the above mentioned polypeptides having hydrophilic side chains lack functional sites for further modifications, limiting their potential uses for bio-conjugation.

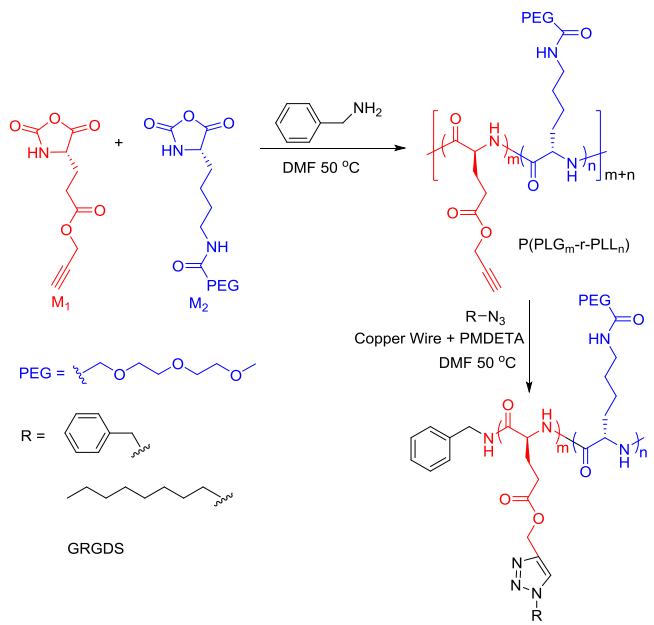
Synthetic polymers have been actively investigated as multivalent ligand scaffolds. Conjugation of bioactive ligands onto the polymers enables control of the size, shape and density of the ligand ensembles.[29] While a majority of the polymers that have been investigated have random coil conformation, we reason that the polypeptides with helical conformations offer several advantages over the random coil counterparts as multivalent ligand scaffolds. First of all, they provide more efficient display of the ligands attached on the side chains relative to the random coil counterparts where some of the side chain moieties are buried in the polymer interior. Secondly, multivalent binding to the random coil scaffold causes uncoiling of the scaffold, resulting in free energy penalty. By contrast, the free energy penalty is expected to be minimal in the



Scheme 1. A schematic depicting the synthesis of non-ionic water-soluble helical polypeptides bearing "clickable" side chains

multivalent binding to the helical scaffold, as the polymers adopt more extended conformation than the random coil counterparts. Before the helical polypeptides can be fully investigated for multivalent scaffold applications, efficient synthetic methods toward non-ionic water-soluble helical polypeptides, which also bear functional side chains to enable conjugation of bioactive moieties, should be developed.

Herein, we report the design and synthesis of a class of non-ionic water-soluble helical-forming polypeptides that can be modified with hydrophobic or hydrophilic moieties on the side chains by copper-catalyzed azide-alkyne cycloaddition (CuAAC) (Scheme 1). The polymers were synthesized by random copolymerization of γ-propargyl L-glutamic acid *N*-carboxyanhydride (PLG NCA) (M1)[17] and N-ε-2-[2-(2-methoxyethoxy)ethoxy]acetyl-L-lysine *N*-carboxyanhydride (EG$_2$-LYS NCA) (M2) (Scheme 2).[1] The propargyl groups on the side chains are expected to enable facile and efficient conjugation of various ligands using click chemistry. The oligomeric ethylene glycol units are expected to confer water solubility and reduce non-specific interactions in cellular environment. The copolymerization method allows for control over the relative solubility in water and the density of "clickable" sites by tuning the feed ratio of the two monomers. It was found that the copolypeptides with less than 50 mol% hydrophobic PPLG segment are readily dissolved in water. Post-polymerization grafting with hydrophobic or hydrophilic moieties can be achieved quantitatively by CuAAC chemistry, yielding non-ionic polypeptide conjugates that retain good water solubility and high percentage of α-helical content. To the best of our knowledge, this is the first study on the synthesis of non-ionic water-soluble helical polypeptides that bear "clickable" side chains through direct ROP of discrete NCA monomers. Preliminary cell culture study reveals that the polypeptide conjugates bearing a integrin binding peptide GRGDS (Gly-Arg-Gly-Asp-Ser) display positive effects in inducing integrin-mediated cell adhesion.



Scheme 2. The synthesis of polypeptide copolymers and post-polymerization conjugation

The monomers (PLG NCA and EG$_2$-LYS NCA) were synthesized by adapting published procedures[1, 17] and purified by column chromatography under anhydrous conditions[30] prior to polymerization. Polypeptide copolymers [denoted as P(PLG$_m$-r-PLL$_n$)] with a targeted degree of polymerization (DP$_n$) of 100 and varying PPLG and PPLL content were prepared by ROP of PLG NCA (M$_1$) and EG$_2$-LYS NCA (M$_2$) in different feed ratios using benzylamine initiators (Scheme 2). All reactions ([M$_1$] + [M$_2$] = 0.4 M, ([M$_1$]+[M$_2$]) : [BnNH$_2$] = 100 : 1) were allowed to proceed at 50 ℃ in DMF under nitrogen and reached nearly quantitative conversion in 12 h. The resulting polymers were isolated by precipitation with diethyl ether and further purified by dissolution in distilled water and centrifugal dialysis. The polypeptide copolymer compositions were analysed by ^{1}H NMR spectroscopy (Table S1). Specifically, the methylene protons (e and j, Figure S1) due to the respective PPLG and PPLL segments were integrated relative to the aromatic protons of the benzyl end-groups (a, Figure S1) to give the polymer composition. The experimentally determined polymer compositions are in good agreement with the theoretical values calculated from the monomer feed ratio and conversion (Table S1). The copolymer molecular weight (M_n) and molecular weight distribution (PDI) were determined by size exclusion chromatography (SEC-DRI) technique. All samples regardless of the polymer composition exhibited mono-modal SEC chromatograms and eluted out at approximately the same elution time, indicating similar hydrodynamic sizes of the polypeptide copolymers. The molecular weight distributions (PDI) are modest in the 1.2 - 1.4 range. There is a small peak at the long elution time, suggesting the presence of low molecular weight species (Figure S3) whose origin is presently unclear. The low molecular weight species can be removed after purification, as evidenced in SEC-DRI (Figure S4). In addition, a shoulder peak appearing at high molecular weight region in the SEC chromatogram of the purified polymer was not observed in the SEC trace of the polymerization reaction mixture. It is attributed to partial solubilisation of polymers after they are completed dried during the workup.

The water solubility of the polypeptides is controlled by the copolymer composition; higher molar fraction of PPLL enhances water solubility, while higher PPLG content lowers the copolymer solubility in water. The P(PLG$_m$-r-PLL$_n$) copolymers with up to 50 mol% PPLG are found to readily dissolve in water (up to around 5.0 mg·mL^{-1} at 25 ℃). A further increase of PLG content to 80 mol% results in polypeptides with negligible water solubility (Entry 4, Table S1). The polymers were also characterized by circular dichroism (CD) and FT-IR spectroscopy to verify the secondary structures in aqueous solution and solid state, respectively. CD

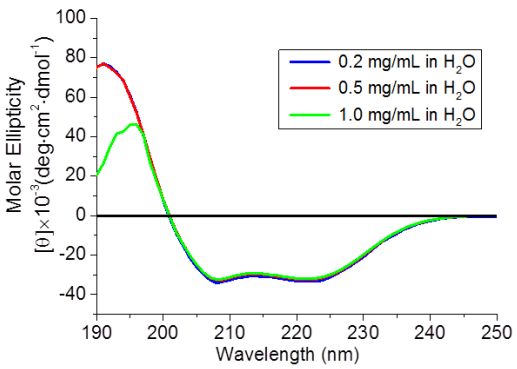


Figure 1. CD spectra of P(PLG$_{14}$-r-PPL$_{72}$) in H$_2$O.

spectra of all polypeptide samples at varying concentrations (0.2-1.0 mg/mL) in water revealed two negative minima at 209 and 222 nm as well as a positive maximum at 190 nm, indicative of primarily α-helical conformations for the polypeptide backbones (Figure 1, S5 and S6). Further spectral analysis by DICHROWEB using Contin-LL revealed that the all polypeptides have significant level of helical conformations in the 84-95% range (Table S2).[31, 32] Moreover, the polymers maintain stable helical conformations within a wide pH range (pH = 1 ~ 13) (Figure S7). In the solid state, the polymers also retain α-helical conformations, evidenced by the strong characteristic amide I (1650 cm^{-1}) and amide II (1536 cm^{-1}) peaks in the FTIR spectrum (Figure S8).[33]

To demonstrate that the water-soluble P(PLG_m-r-PLL_n) polypeptides can be further modified with hydrophobic moieties by CuAAC chemistry to afford water-soluble conjugates, P(PLG_m-r-PLL_n) was treated with 1.3 equivalents of octyl azide or benzyl azide in the presence of copper wire[34] and 1.3 eqv. of PMDETA ligand at 50 ℃ in DMF for 24 h. ^{1}H NMR spectra of the resulting polymers [denoted as P((PLG_m-g-Oct)-r-PPL_n) and P((PLG_m-g-Bn)-r-PPL_n)] revealed a new peak at 8.1 ppm (d, Figure 2), which is characteristic of the triazole proton formed from the CuAAC reaction. The grafting of the hydrophobic moieties is quantitative (Figure 2), as evidenced by the complete disapperance of the methylene protons due to non-modified PPLG segment (c, Figure 2) at 4.8 ppm and the appearance of the same protons at 5.1 ppm (c', Figure 2) resulted from the successful formation of the triazole group. These reactions suggests that P(PLG_m-r-PLL_n) copolymers can be grafted with different hydrophobic molecules for targeted applications.

For hydrophobically modified polypeptides, it is important to assess the water solubility of the resulting conjugates. It was found that both P((PLG_{17}-g-Oct)-r-PPL_{69}) and P((PLG_{17}-g-Bn)-r-PPL_{69}) can be dissolved in water at concentrations up to 2.0 mg·mL^{-1}. This indicates that conjugation to P(PLG_m-r-PLL_n) polymer is effective in enhancing the water solubility of small hydrophobic molecules to a significant concentration. Dynamic light scattering analysis of a P((PLG_{17}-g-Oct)-r-PPL_{69}) solution in water revealed a mono-modal particle size distribution with a hydrodynamic diameter of 18.8 nm (PDI = 0.205). It is consistent with the calculated end-to-end for P((PLG_{17}-g-Oct)-r-PPL_{69}) polymer (12.9 nm), assuming the polymer adopting a helical rod conformation (Figure S17). CD analysis of the resulting conjugates revealed that both P((PLG_{17}-g-Oct)-r-PPL_{69}) and P((PLG_{17}-g-Bn)-r-PPL_{69}) retain α-helical conformations with residual molar ellipticities comparable to that of the parent polypeptide in water in the 0.2 – 1.0 mg·mL^{-1} concentration range

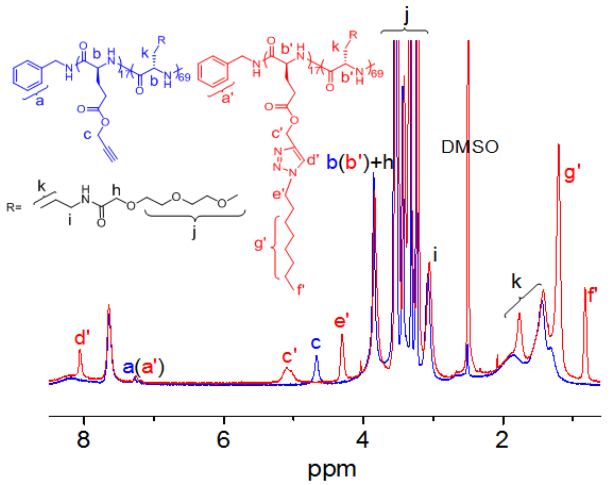


Figure 2. ^{1}H NMR spectra of P(PLG_{17}-r-PPL_{69}) and P((PLG_{17}-g-Oct)-r-PPL_{69}) in DMSO-d_6.

(Figure S9 and S10). Grafting of octyl and benzyl side groups do not appear to disrupt the backbone conformations, as the helical content of the hydrophobically modified polypeptides remains high (93% and 84% respectively) as compared to that of the parent polypeptide (85%) (Table S2). The non-disturbed helical conformations are desirable for many biomedical applications such as multivalent ligand platform, where the grafted hydrophobic moieties on the helical surface can be more readily accessed than the random-coil counterpart.

To further demonstrate that the sidechain grafted polypeptides are effective carriers for biologically active moities, an azido-terminated GRGDS pentapeptide which is known to induce integrin-mediated cell adhesion[35] was conjugated to the polypeptide P(PLG_{14}-r-PLL_{72}) by CuAAC chemistry. The conjugation yielded a GRGDS-polypeptide conjugate P((PLG_{14}-$GRGDS_8$)-r-PLL_{72}) with 9 mol% GRGDS sites along the helical backbone (Figure S11). CD analysis indicates the conjugate retains high level of helical conformation (99%) in aqueous solution (Figure S12 and Table S2). Preliminary cell adhsion studies (Figure S13) revealed that GRGDS-polypeptide conjugate can promote the adhesion of Chinese hamster ovary (CHO) cells, though less effective than the natural ligand fibrinogen. By contrast, the parent polypeptide without GRGDS was unable to promote any cell adhesion. This result suggests that GRGDS moieties on the helical polypeptide side chains are not cloaked by the oligo(ethylene glycol) side chains and can be accessed by the cell membrane-bound receptors, resulting in cell adhesion.

In conclusion, we have demonstrated a successful synthetic route towards a new class of non-ionic water-soluble "clickable" helical polypeptides by organo-mediated copolymerization of discrete NCA monomers. The polypeptides maintain stable α-helical conformations in aqueous solution in a wide pH range (pH = 1 ~ 13) and in solid state. The copolymerization strategy produces polypeptides having tunable densities of "clickable" sites which allow for further conjugation of hydrophobic or hydrophilic moieties. It was shown that the hydrophobically and hydrophilically-modified polypeptide conjugates retain high level of α-helical conformations. The water solubility of the conjugates is strongly dependent on the relative hydrophobic and hydrophilic content in the conjugates. We envision the potential use of the helical polypeptides as multivalent ligand scaffolds. The helical polypeptides are expected to offer more efficient ligand display and reduced entropy penalty upon multivalent binding relative to the random coil counterparts. These aspects will be the focus of future investigation.

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Notes and references

[a] Department of Chemistry and Macromolecular Studies Group, Louisiana State University, Baton Rouge, Louisiana 70803, United States. [b] Department of Biological Sciences, Louisiana State University, Baton Rouge, Louisiana 70803, United States. E-mail: dhzhang@lsu.edu, Fax Number 01-575-225-3458

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