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Synthesis and Noncovalent Protein Conjugation of Linear-Hyperbranched PEG-Poly(glycerol) α,ω_n-Telechelics

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The application of proteins and peptides as human therapeutics is developing rapidly with the discovery of novel pharmaceutically active peptides and proteins.[1] A drawback of protein-based biotherapeutics is their fast destruction in both the digestive system and the circulatory system; i.e., they are rapidly removed by proteolytic digestion and renal excretion.[2] In the past decade the (covalent) attachment of synthetic macromolecules has proven to be an efficient way to improve protein stability with reduced immunogenicity and extended plasma half-life times.[3] Additional physical properties can be introduced, such as photo-, temperature-, or pH-sensitivity. A recent review gives an excellent overview on the state of the art in this field.[4]

Poly(ethylene glycol) (PEG) is probably the most frequently used biocompatible polymer in everyday life and also in medicine, where the "PEGylation" of proteins is an extensively used fundamental strategy designed to improve the biochemical and pharmacological properties of therapeutics as mentioned above.[5] In recent works it has been demonstrated that branched PEG structures with a lysine branching unit located directly at the protein lead to improved circulation times and are thus superior compared to linear PEG chains.[6,7] A potential drawback in currently used PEGylation strategies is the lack of additional functional groups for further derivatization in the conjugated polymer. On the other hand, heterotelechelic PEGs have been a focus of PEG chemistry for decades. Various functional groups can be introduced by functional initiation or termination of the anionic ROP of ethylene oxide or by modification of the terminal OH group.[8]

Hyperbranched poly(glycerol) (*hb*PG) has similar toxicological properties as PEG[9] but, in addition, offers the possibility for introduction of further structural elements after conjugation to proteins due to its polyfunctionality. In this communication, we describe the first example of linear-hyperbranched α,ω_n-telechelic block copolymers, consisting of a linear PEG block and a *hb*PG block, in which the polyfunctionality ω_n can be adjusted by the degree of polymerization (DP_n) of polyglycerol (OH groups). The synthesis of various other linear-hyperbranched block copolymers has been reported by our group.[10] Furthermore we demonstrate the noncovalent conjugation of the polymers to avidin via the distinct α-functionality.

To obtain well-defined, precisely monofunctional hyperbranched polyglycerol-based structures for bioconjugation, a new strategy had to be devised (Figure 1). For the targeted synthesis an initiator for EO polymerization was required that tolerates both strongly basic oxyanionic and acidic acetal cleavage conditions, as required for our synthetic pathway. For the introduction of the amino functionality in the α-position we applied di(benzyl)aminoethanol as a new initiator, which is easily obtained from ethanolamine and benzyl bromide in good yields, but is also commercially available. The benzyl protective groups can be removed quantitatively without chain scission or other side reactions after polymerization by catalytic hydrogenation. The synthesis of the α,ω_n-telechelics is based on a straightforward four-step protocol (Figure 1, Table 1): (i) anionic polymerization of EO and/or EEGE with a novel protected functional initiator; (ii) acidic hydrolysis of the acetal protective groups, (iii) (hyper)grafting of glycidol, (iv) hydrogenation to generate the amine in the α-position. Molecular weights of linear and hyperbranched blocks were systematically varied, and narrow molecular weight distributions were obtained in all cases (cf. Table 1, $M_w/M_n < 1.2$). The resulting α,ω_n-heterotelechelic block copolymers cover a wide range of molecular weights, block ratios, and functionalities. By variation of the PEG chain length, the distance between the shielding *hb*PG-block from the functional protein can be adjusted very accurately. The PEG spacer can also be omitted in the case of the synthesis of novel α,ω_n-hyperbranched PG homopolymers, if required. Via variation of the DP_n of polyglycerol, the steric bulk of the globular, hyperbranched block can be further adjusted.

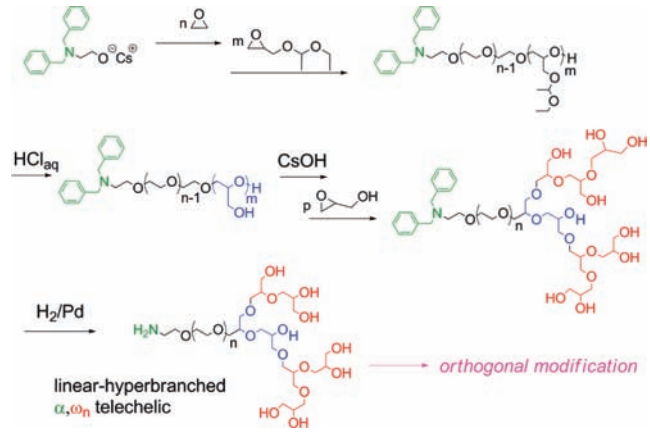

Figure 1. Synthetic scheme of the preparation of α,ω_n-linear-hyperbranched heterotelechelics, carrying precisely one single amino function and *n* terminal hydroxyl groups.

Selective transformation of the single α-amino group of the *lin-hb* α,ω_n telechelics in the presence of multiple hydroxyl termini is a synthetic challenge. However, this functionality can be addressed with high specifity by pentafluorophenyl (PFP) active esters, and a variety of functional moieties can thereby be introduced quantitatively. Biotine attachment at PEG-*b*-PG via the amine in the α-position is realized by quantitative conversion into an amide, using the pentafluorophenyl active ester of biotin.

As proof of principle, the biotinylated PEG-*b*-PGs were conjugated in a noncovalent manner with avidin and streptavidin, respectively. Avidin was chosen as the model protein for two reasons: (a) Avidin

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possesses an extraordinarily high affinity to biotin (affinity constant $\sim 10^{15}$ M), and each of its four subunits can bind exactly one molecule of biotin. (b) The avidin−biotin system has been studied as a model compound in protein−ligand interaction[11] and for a large number of applications. For instance tumor pretargeting,[12] improved clinical diagnostics,[13] and protein labeling and proteomics are based on this system. Due to its extraordinary stability it is also applied as a research tool in surface engineering, supramolecular protein chemistry, and drug delivery systems.[14] Recently, attachment of linear polymers or dendrons to avidin has been reported in some works.[15]

Table 1. Molecular Weight Data of $\alpha-\omega_n$ Heterotelechelics

no.	compound	M_n[a]	PDI[a]	M_n[b]
1	Bn_2NPEG_{25} *lin*PG_5	1500	1.06	1400
2	Bn_2NPEG_{120} *lin*PG_{15}	6300	1.05	6500
1a	Bn_2NPEG_{25} *hb*PG_{20}	2400	1.14	2700
1b	Bn_2NPEG_{25} *hb*PG_{50}	4700	1.23	5200
2a	Bn_2NPEG_{120} *hb*PG_{70}	10 000	1.19	10 600
1a*	H_2NPEG_{25} *hb*PG_{20}	2200	1.15	2600
1b*	H_2NPEG_{25} *hb*PG_{50}	4700	1.25	5200
2a*	H_2NPEG_{120} *hb*PG_{70}	10 200	1.18	10 600
3	Bn_2N*lin*PG_{15}	1200	1.17	1300
3a	Bn_2N*hb*PG_{90}	6700	1.20	6800
3a*	H_2N*hb*PG_{90}	6400	1.21	6400

[a] Determined via size-exclusion chromatography in dimethylformamide vs polystyrene standards. [b] Determined via ^{1}H NMR spectroscopy.

For the noncovalent bioconjugation with avidin we chose the PFP-ester of biotin that is readily synthesized from biotin and pentafluorophenol in excellent yields (95%, cf. Supporting Information, SI). The coupling reaction with the polymer is conducted at ambient temperature with a slight excess of the PFP-ester. Subsequent purification is achieved by precipitation or dialysis against methanol. Quantitative modification of the polymer with biotin was confirmed via MALDI-ToF mass spectrometry and NMR spectroscopy (SI).

Bioconjugation of the biotinylated polymers was studied by the displacement reaction of 2-(4-hydroxyphenylazo)benzoic acid (HABA) and monitored via UV/vis spectroscopy. Avidin forms a red complex with HABA that absorbs at ca. 500 nm (see inset in Figure 2). The binding affinity of the HABA−avidin complex (dissociation constant $K_d = 10^{-6}$ M) is considerably lower than the affinity of avidin to biotin ($K_d = 10^{-15}$ M). After addition of the biotinylated polymer, the red complex is destroyed and the characteristic UV-absorption of free HABA is observed, confirming the successful polymer complexation by avidin. In addition, HPLC measurements of the noncovalently conjugated avidin show a strong shift to higher elution times compared to native avidin (Figure 2). This clearly confirms that the complex between avidin and the biotinylated α,ω_n-structure has been formed.

In summary, we present the first synthesis of well-defined α,ω_n-linear-hyperbranched PEG-PG heterotelechelics with a single amino moiety in the α-position and subsequent attachment of biotin in this position. The structure of the functionalized α,ω_n-block copolymers was confirmed via MALDI-ToF spectroscopy. This material was subsequently used for noncovalent bioconjugation, which can be achieved with or without a linear PEG-spacer. We believe that the combination of a functional polymer synthesis and subsequent quantitative modification via PFP-esters is a general approach for the introduction of a variety of functional groups and also covalent bioconjugation with other proteins and peptides. Currently, the biological and biomedical implications of this approach as well as covalent protein attachment to the α,ω_n telechelics are under investigation.

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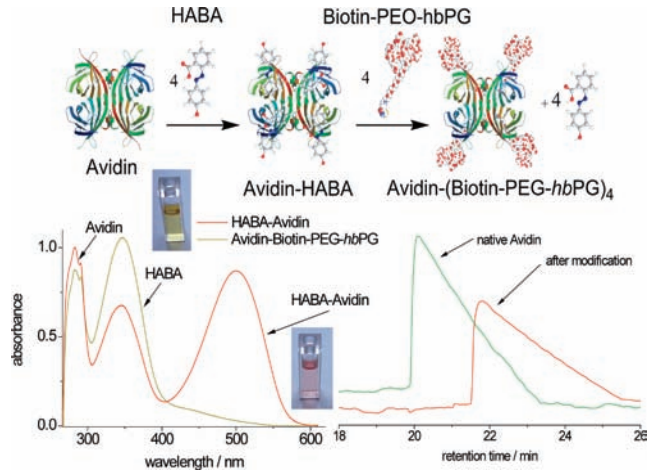

Figure 2. *Top:* Scheme of the noncovalent conjugation of avidin with 2-(4-hydroxyphenylazo)benzoic acid (HABA) and subsequent displacement by the biotinylated polymer. *Bottom, left:* UV/vis spectra of the HABA−avidin complex (red) and after addition of the biotinylated polymer (yellow, characteristic absorption of HABA). *Bottom, right:* HPLC diagram of native avidin and the noncovalently modified protein.

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Supporting Information Available: Additional SEC curves, NMR, MALDI-ToF and HPLC data. This material is available free of charge via the Internet at http://pubs.acs.org.

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