Linking Poly-L-arginine To Poly-DL-lysine By Using The Maillard Reaction: A Block Copolymer That Binds The Cluster Anion of Copper and Penicillamine

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Pentose sugars, such as ribose and arabinose, easily graft polylysine to polyarginine by means of the Maillard reaction, resulting in water soluble or insoluble block copolymers, depending on the starting concentrations of the reactants. Soluble block copolymers prepared by this method have a molecular weight of no lower than 200,000 g/mol, whereas the original homopeptide molecular weights were less than 50,000 g/mol. The water soluble copolymer complexes with the red-violet anionic cluster of copper and penicillamine at pH 8.8, although with progressive dissociation into the unaltered copolymer and unaltered cluster anion, whereas suspensions of an insoluble version of the copolymer, with the lysine/arginine molar ratio near 30/70, exhibited a strong affinity for the same copper cluster. Because of its lysine content, a copolymer of the latter type is easily biotinylated. Biotinylated and complexed with the copper cluster, the copolymer might be of interest as a biologically selective carrier for delivering copper radionuclides by using affinity targeting methods. ***© 2003 Oklahoma Academy of Science***

INTRODUCTION

The beta-emitting radionuclides ^{64}Cu and ^{67}Cu are suitable for radioimmunotherapy of malignant cells as a result of their short half-lives and their relatively short range particle radiation. Early experimental radioimmunotherapies have considered these copper nuclides (DeNardo et al 1999, 1998; Kukis et al 1994), which are preferable to iodine nuclides because radioiodine threatens thyroid tissue. Regardless of the nuclide species being considered, the strength of nuclide bonding to the targeting antibody is a central issue.

The red-violet cluster anion of D-penicillamine and copper (RVC) (Birker et al 1981) is of interest to copper-targeting schemes because even strong copper chelating resins, such as Chelex-100, do not remove its copper. RVC might be a suitable copper carrier if crosslinking strategies can be developed. The RVC anion has the formula $[Cu(I)_8Cu(II)_6L_{12}Cl]^{5-}$ (where L is D-penicillamine). Its core structure is shown in Fig. 1 (Birker et al 1981), and its synthesis and purification is fast and straightforward (Cooke et al 1983). The stability of RVC has been examined in some detail (Giles et al 1987). Experiments with rabbits revealed that RVC did not produce detectable toxic effects when it was injected intravenously as the sodium salt, and its renal clearance was rapid, similar to that of inulin (Bergevin et al 1984).

In previously published work (Hertzler et al 2000) it was found that RVC interacted significantly with poly-DL-lysine (PDLL), and the lysine polymer is easy to biotinylate, thus is suitable for linking to antibodies. The PDLL binding (association) constant for RVC is sufficient to be the basis of a microscale colorimetric immunoassay (Hertzler et al 2000) based on RVC's strong absorption of visible light (wavelength at maximum = 520 nm, molar extinction coefficient ~28,000 $M^{-1}cm^{-1}$), but at physiological pH the binding (association) constant, about 10^5, is not strong enough for a radioimmunotherapy. The purpose of the

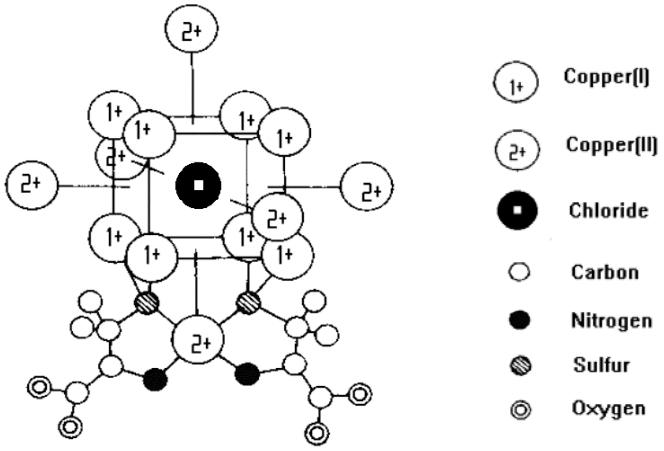

Figure 1. Core structure of the mixed valence, red-violet cluster *anion* of copper and penicillamine (RVC). Atom types are identified in the key beside the structure. The chloride ion is *central*, surrounded by a cube of eight Cu(I) ions. Six Cu(II) ions are above the cube faces. This is cubo-octahedral pseudosymmetry due to stabilization distortion and the asymmetry of the optically active ligand. The figure shows two of twelve ligands in the $[Cu(I)_8Cu(II)_6L_{12}Cl]^{5-}$ anion structural unit, where the ligand (L) is penicillamine. Copper oxidation states are noted. The penicillamine sulfur (thiolate) atoms bridge between two Cu(I) ions and one Cu(II) ion, and two amine nitrogens complete the N,N,S,S coordination around each Cu(II). Hydrogen atoms are not shown.

ongoing investigation is to identify better carriers with potential for linking copper to antibodies.

In the preliminary part of this work, several cationic synthetic peptides were screened for RVC binding effects, and of the tested materials, poly-L-ornithine possessed RVC binding properties comparable to PDLL, confirming our expectations because of poly-L-ornithine's close structural relationship to PDLL. Poly-L-arginine (PARG) interacted strongly. An actual binding (association) constant was not measured, but it is very large at neutral pH because no reversal of binding was observed. PARG forms an electroneutral complex with RVC on cellulose polyacetate electrophoresis strips and remains stationary at length, without decomposing.

Because PARG is not easily biotinylated, a block copolymer of PDLL and PARG might combine better RVC-binding properties with ease of biotinylation. In part, this proved to be the case. The intent is to create an adapter for attaching RVC to avidin-linked antibodies.

MATERIALS and METHODS

The crosslinking method chosen for block copolymerization is the Maillard reaction (Grandhee and Monnier 1991), in which pentoses, such as ribose or arabinose, react specifically with the pendant groups of lysine and arginine under alkaline conditions and in the presence of a small amount of molecular oxygen to form covalent links between peptide chains. Hexoses and hexose derivatives, such as glucose, fructose, and ascorbate also led to crosslinks (Grandhee and Monnier 1991). The focus of this work has been on using the Maillard reaction, thought to be involved in natural biological aging processes, as a synthetic method, i.e., for peptide-peptide conjugation. The use of polylysines as an adapter for constructing pharmacologic carriers has already been established (Clegg et al 1990).

The two homopolymers used to prepare an arginine/lysine block copolymer were obtained from Sigma Chemical Company, P.O. Box 14508, St Louis, MO 631778. For these two polymers the mean degree of

TABLE 1. Electrophoretic detection of RVC interactions with synthetic peptides

Substance (Sigma Number)	Interaction	Migration
poly(D-glu,D-lys)6:4 (P-7658)	none	-
poly(L-his, L-glu)/poly-DL-alanine/ poly-L-lys (M-3774)	none	-
poly-L-tyrosine (P-7887)	none	-
poly(L-tyr, L-glu)/poly-DL-ala/poly-DL-lys (M-3649)	none	-
poly-DL-ala/poly-L-lys (M-3524)	none	-
poly(L-lys, L-ala, L-glu, L-tyr) 5:6:2 (P-1278)	decomposing complex	0.6
PDLL (P-9011)	moderately strong	-1.5
poly-L-ornithine (P-2533)	moderately strong	-0.6
PARG (P-7762)	very strong	0.0

polymerization (DP) is 196 in the case of PDLL (Sigma P9011, about 25,000 g/mol), and 287 for PARG (Sigma P7762, about 45,000 g/mol). The crosslinking procedure consists of the following steps: 10 mg PDLL and 10 mg arabinose are dissolved in 10 mL pH 9.0 phosphate buffer to obtain solution A. Then a second solution (B) is prepared of 10 mg PARG in 10 mL pH 9.0 phosphate buffer. Desired proportions of A and B for a total volume of 1.00 mL are mixed thoroughly and kept at 65°C for up to 48 h in capped 2-mL Eppendorf-type plastic microcentrifuge tubes. A molar ratio may be calculated from known amino acid molecular masses and the above quoted DP values, e.g., 1.78 times the PDLL/PARG mass ratio. The proportions of A and B were varied over a wide range, and a mass ratio of 0.25 (0.20 mL of A and 0.80 mL of B) is close to the optimal 30/70 molar ratio found for PDLL/PARG.

Use of a capped tube limits the amount of available oxygen. The mixture turns pale brown as the reaction proceeds. Some of the seals failed in these syntheses, leading to a partial loss of solvent and a higher reactant concentration, but that had the fortuitous result of providing an insoluble version of the block copolymer.

Zone electrophoresis of the soluble reaction product was carried out on cellulose polyacetate strips (Sepraphore III) in phosphate buffer and were stained with amido black (Ouchterlony 1973). Amido black stain is a disulfonic acid and, thus, reacts with lysine, arginine, and histidine pendant groups.

RESULTS and DISCUSSION

Table 1 summarizes results for nine peptides tested for binding affinity toward RVC. In these experiments the peptide in question was dissolved in a buffered aqueous solution containing RVC and subjected to zone electrophoresis. The tabulation shows results obtained in a pH 5 acetate buffer, but measurements were also obtained in pH 7.5 phosphate buffered saline. The first five peptides of Table I showed no electrophoretic evidence of interaction at pH 5.0 or 7.5.

The table's third column "migration" describes electrophoretic migration relative to anionic RVC, where 1.0 is the rate of migration of the free RVC anion, and 0.5 describes an anionic complex migrating half as fast as free RVC. Negative values describe complexes with net cationic charges. Four of the peptides formed complexes with RVC. The table's sixth entry, poly(L-lys,L-ala,L-glu,L-tyr) 5:6:2 produced a decomposing *anionic* red-violet band trailing unbound RVC at relative migration +0.6, but only at pH 5.0. At pH 5.0 (acetate buffer) RVC associates with PDLL and poly-L-ornithine, forming net *cationic complexes* with relative migrations of -1.5 and -0.6, respectively. The PDLL/RVC complex is especially stable at pH 5.0, persisting for more than 30 min of electrophoretic migration (Hertzler et al

2000). At pH 7.5 (phosphate buffered saline) the PDLL complex showed a relative *cationic* migration of -0.9 but decomposed rapidly (~5 min), whereas no complex was seen at this pH in the case of poly-L-ornithine. At each of these two pH values the electroneutral complex of PARG with RVC remained fast at the origin (migration 0.0). This observation led us to consider block copolymers of PARG and PDLL, which are not available commercially, as easily conjugated carriers for RVC.

Evidence showing that the Maillard reaction is taking place in reacting mixtures of PARG, PDLL, and arabinose includes the characteristic browning (Grandhee and Monnier 1991). The product also moves with the void volume on thin layer Sephadex G-200 plates, confirming a molecular weight increase to at least 200,000 g/mol, compared to less than 50,000 g/mol for each of the two homopolymers before crosslinking. As shown in Fig. 2, the soluble reaction product is electrophoretically distinct from PDLL or PARG on cellulose polyacetate strips, and there are no detecable residuals of either of the two starting reactants.

The soluble block copolymer does interact with the copper cluster. RVC pigmentation associated with the block copolymer band, but at pH 8.8 in a borate buffer the net migration was anionic (about 0.2 relative to RVC alone) and showed a progressive dissociation. At pH 7.5 the complex is virtually electroneutral and shows decomposition. Because the soluble block copolymer/RVC complex decomposes during electrophoresis, it is not a significant improvement over the PDLL/RVC complex.

In contrast, the insoluble block copolymer possesses an enhanced binding affinity for RVC. A block copolymer suspension prepared with a 30/70 lysine/arginine molar ratio interacted strongly with the RVC. This interaction is pH dependent and very strong in the pH range between 5.0 and 8.8, which include the physiological pH range. Specifically, RVC/block copolymer affinity was observed at pH 5.0 (acetate buffer), 7.5 (phosphate buffered saline) and 8.8 (borate buffer). The red-violet pigmentation of RVC is easily visualized even in the micromolar range. Once attached to a finely divided aqueous suspension of the solid copolymer, RVC pigmentation is not removed by repeated washings with phosphate buffered saline, pH 7.5, made up as 0.120 M NaCl, 0.0027 M KCl, and 0.010 M phosphate. RVC apparently saturates strong binding sites. Binding effects for RVC are observed up to a 70/30 lysine/arginine molar ratio, beyond which the behavior is much like PDLL itself (i.e., the pigment washes out). Water

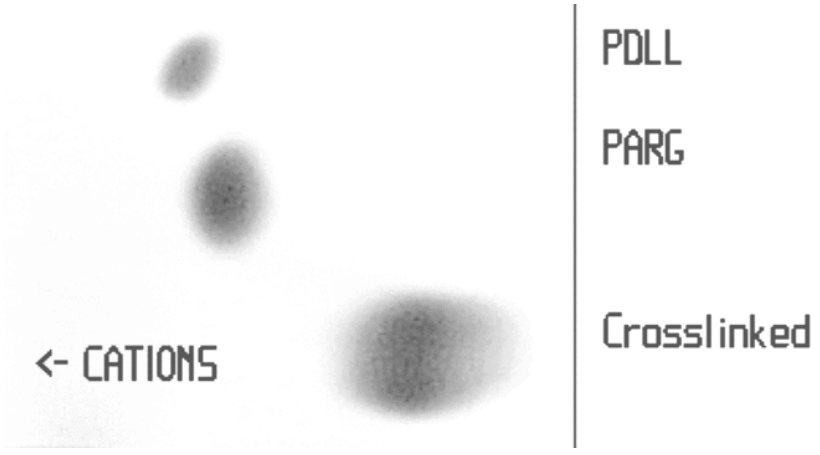

Figure 2. Electrophoresis comparison of PDLL, PARG and the PDLL/PARG block copolymer. The vertical line is the origin. The Maillard reaction product (crosslinked) is electrophoretically distinct from PDLL and PARG, although all three materials migrate as cations. The crosslink product, i.e., the block copolymer, shows a distribution (elongated band).

soluble PDLL exhibits significant interaction only at or below pH 5 (Hertzler et al 2000). Block copolymer suspensions used in these procedures sediment rapidly at 8,000 rpm in the Eppendorf microcentrifuge (model 5415), indicating particle sizes larger than 10 µm.

Molecular oxygen is the oxidant in the variant of the Maillard reaction used here. Degree of polymerization control is lost if the tube seal fails, leading to evaporation, increased reactant concentrations (above a gel point) and an insoluble high polymer product matrix. Some tube caps simply did not seal well. The material is also more prone to precipitate at reaction pH values below 8 (a pH 8.8 borate buffer favors formation of a soluble polymer). Reproducibility was thus not especially good between synthetic batches.

Only the insoluble copolymer is of interest as an RVC carrier. Fine solid suspensions are not necessarily an obstacle to antibody mediated delivery schemes. Solids may be fragmented to stable suspensions or even colloids by means of ultrasonic irradiation, then crosslinked to antibodies in that form. If biotin survives the Maillard reaction conditions, the preferred method might be to biotinylate PDLL first (Hertzler et al 2000) and then carry out a block copolymerization with PARG.

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