Biomaterials

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Saccharide–Peptide Hybrid Copolymers as Biomaterials**

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Biomaterials are important for many biomedical applications such as implants and prosthetics, pharmaceutical formulations, drug and gene-delivery agents, DNA and protein microarrays, and tissue engineering.[1] Synthetic polymers remain the most versatile class of biomaterials because of the ease in controlling their compositions, structures, and properties.[2,3] There is currently a great demand for novel polymeric biomaterials with tailored structures and more-defined functions. Ideally, desired biomaterials can be chemically synthesized in a ground-up approach to exhibit precisely the needed chemical, biological, and engineering properties for the targeted medical application.

An important strategy for designing new biomaterials is to construct synthetic polymers from natural building blocks. The premise is to combine the advantages of both bio- and synthetic polymers, thereby gaining the biocompatibility and biodegradability of natural materials and the versatility of synthetic structural design. Examples of biomaterials made

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from natural building blocks include poly(lactic acid),[4] poly(glycolic acid),[5] poly(anhydride)s,[6] poly(amino acid)s,[7] pseudo-poly(amino acid)s,[8] carbohydrate-derived polyesters,[9] and artificial proteins.[10] Whereas these polymers generally exhibit good biocompability, and some are used in important clinical applications, their structural diversity and functional properties are relatively limited, warranting further development of more versatile biomaterials.

Herein we describe a new class of biomaterials derived from natural saccharide and amino acid building blocks (Figure 1). Our design is based on the following considerations; 1) saccharides and amino acids are abundant and readily available natural monomers, 2) these natural building blocks are likely to yield polymers with inherent biocompatibility that then degrade into natural, nontoxic, and biosorbable species, 3) their rich functionalities allow convenient modification/tailoring of materials for desired applications, and 4) the modular synthesis offers advantages in combining structural precision with design flexibility.

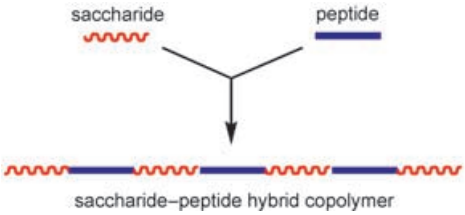
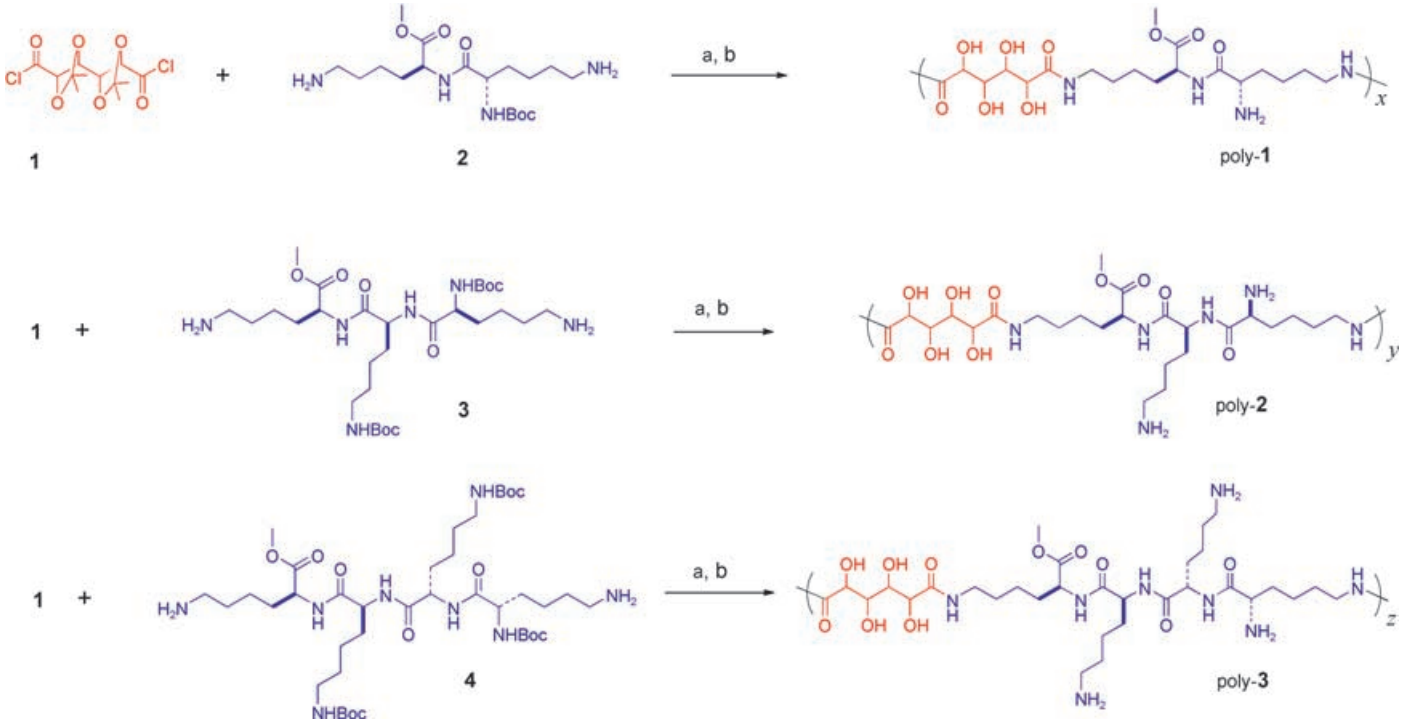


Figure 1. Formation of the saccharide–peptide hybrid copolymer.

The combination of various modules gives rise to numerous polymers suitable for combinatorial screening of the chemical, biological, and mechanical properties of these polymers. Although much effort has been devoted to making oligomeric glycopeptides that can mimic the structure and function of natural glycoproteins,[11,12] little has been reported on the synthesis of high-molecular-weight saccharide–peptide polymers.[13,14] In our novel design, both the saccharide and peptide building blocks are incorporated into the main chain of the polymer. Herein we report our general design concept, synthesis, and the initial testing of a new class of biomaterials. We also discuss our studies of these saccharide-peptide hybrid copolymers in one specific biomedical application, gene delivery.

We chose oligolysines and a galactose-derived monomer **1** for copolymerization. Three hybrid polymers (poly-**1,** poly-**2**, and poly-**3**) were synthesized by interfacial polymerization with various peptide monomers **2**–**4** (Scheme 1). ^{1}H and ^{13}C NMR spectroscopic analyses were used to confirm the structure of each polymer. The number-averaged molecular weights (M_n) of the three prepolymers for poly-**1**, poly-**2**, and poly-**3** were measured by gel-permeation chromatography with polystyrene calibration standards to be 15000, 9085, and 13200 g mol^{-1} respectively. Global cleavage of the acetonide and *tert*-butoxycarbonyl (Boc) protecting groups afforded the final carbohydrate–peptide hybrid copolymers, poly-**1**, poly-**2**, and poly-**3** (details of the synthesis and characterization can be found in the Supporting Information).

After the successful synthesis of the hybrid copolymers, we investigated their general properties as biomaterials including the biodegradability, cytotoxicity, and immunological properties. Enzymatic-degradation studies with serine proteases (subtilisin A and trypsin) showed that the polymers are indeed biodegradable. MALDI-TOF mass spectra were measured periodically to monitor qualitatively the decrease



Scheme 1. Synthesis of galactaro–oligolysine hybrid copolymers (poly-**1**, poly-**2**, poly-**3**). a) Interfacial polymerization, H_2O/CCl_4; b) 30% trifluoroacetic acid in THF/water.

in the molecular weight of the polymer over time. The resultant profile indicated that the polymers were nearly completely degraded after 5–7 days of exposure to an enzyme (see Supporting Information).

The cytotoxicity of the polymers was then assayed at various concentrations by means of a standard MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) test. The hybrid copolymers exhibited minimal cytotoxicity to Cos 7 cells at a range of concentrations (Figure 2). Poly-L-lysine (PLL) with $M_n = 8500\ g\,mol^{-1}$ was used as a control

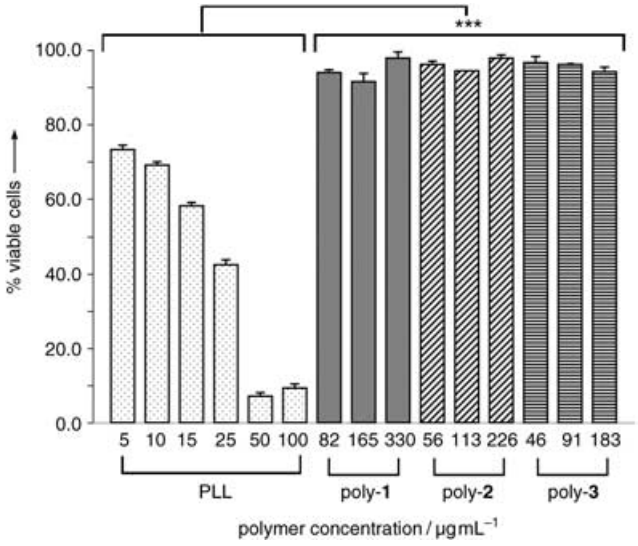


Figure 2. Cytotoxicity data acquired through MTT testing at various polymer concentrations with PLL as the control. Comparison was made with cationic amino groups at equivalent molar concentrations, consistent with those used in gene transfection studies. Standard deviations are shown with error bars ($n = 4$). The symbol *** indicates statistical significance at levels of $p < 0.001$ for the experimental polymers versus each concentration of PLL (indicated by brackets). The p values were obtained by using Student–Newman–Keuls multiple comparisons testing.

because our polymers are derived partially from L-lysine and are of comparable molecular weight, which affects both cytotoxicity and transfection levels.[15] Comparisons between the polymers and the control were made at equivalent molar concentrations of cationic amino groups (the same as those used in later gene-transfection studies). The contrast is striking: whereas the PLL homopolymer exhibits high cytotoxicity at relatively low concentrations (5–100 $\mu g\,mL^{-1}$), our hybrid copolymers show lower cytotoxicity even at higher concentrations (82–330 $\mu g\,mL^{-1}$ for poly-**1**; 56–226 $\mu g\,mL^{-1}$ for poly-**2**; and 46–183 $\mu g\,mL^{-1}$ for poly-**3**). The placement of saccharide spacers on the main chain of the polymers lowered their cytotoxicity to levels approaching the blank controls in the absence of polymer. Although the exact mechanism for this decrease in toxicity remains to be investigated, we believe that the breakage of the cationic polypeptide into short segments with saccharide spacers lowers continuous charge density while the hydrophilic saccharide fragments shield the surface charge of the polyplexes. Both effects are hypothesized to alleviate disruptive coulombic interactions of the polyplexes with the cell membrane. Other studies have shown that the introduction of carbohydrate units into other polymers also results in a lower cytotoxicity.[16]

As our carbohydrate–peptide copolymers are new compounds, it is important to test whether they generate immune responses in vivo. As a representative example, the immunogenicity of poly-**3** was evaluated by ELISA with Fisher 344 rats as models. Based on a standard protocol, 100 μg of poly-**3** was administrated in the first, third, and sixth weeks by either subcutaneous (SC) injections in the footpad or by intravenous (IV) injections into tail veins. The animals underwent a phlebotomy 21 days after each administration of the polymer. The serum for ELISA testing was obtained at three-week intervals. If a positive immunogenic response is elicited by the polymer, then antibodies generated in the rat serum would bind to the polymer-coated wells. An anti-rat immunoglobulin (IgG) conjugated HRP (horseradish peroxidase) then binds to the adsorbed polymer antibodies, catalyzing oxidation of a substrate that can be detected by UV/Vis spectrometry (see Supporting Information for experimental details). Figure 3 summarizes the ELISA data, which show no evidence of antibody response. All rats were healthy (no weight loss, normal activity, good hygiene/quality fur), which suggests that there is no adverse immune response or toxicity.

After the assays to test their biodegradability, cytotoxicity, and immunogenicity, the hybrid copolymers were evaluated for a specific biomedical application. Synthetic cationic

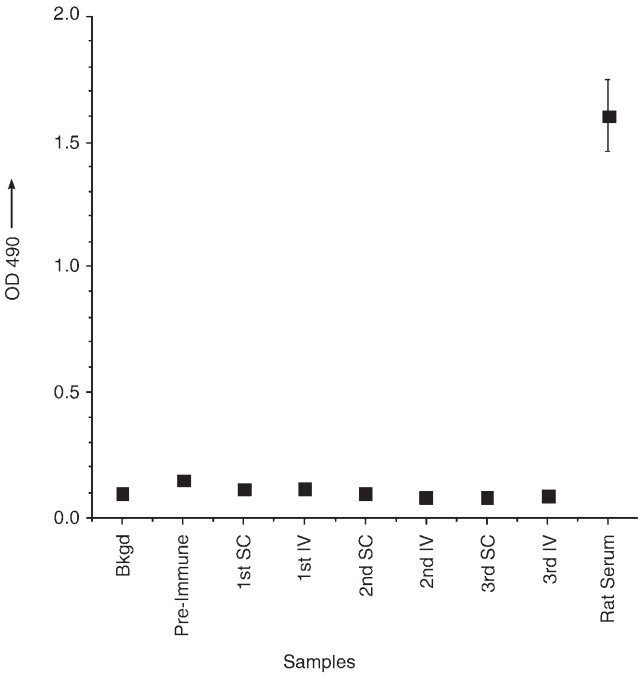


Figure 3. Anti-polymer ELISA data, which include the results for background (Bkgd), pre-immune serum, subcutaneous (SC), intravenous (IV), and normal rat serum (positive control: the wells were coated with rat serum enriched in antibodies, hence resulting in an expected positive signal). The y axis is the optical density (OD) at $\lambda = 490$ nm for the oxidation product of ELISA substrate, tetramethylbenzidine (TMB), which represents the level of immunogenic response of the polymer. The first, second, and third sera were taken at the third, sixth, and ninth week, respectively ($n = 3$). All experimental details are included in the Supporting Information.

polymers are currently being actively pursued as gene delivery vectors because they can neutralize and condense DNA into particles capable of undergoing endocytosis.[17] A critical issue in developing effective synthetic polymeric vectors is that competent gene carriers, such as PLL and polyethyleneimine (PEI), are often cytotoxic.[17,18] As our hybrid copolymers carry cationic charges at physiological pH values, and have minimal cytotoxicity, they were evaluated as vectors for gene delivery. Electrophoretic mobility-shift assays (EMSAs) indicated that poly-**1**, poly-**2**, and poly-**3** efficiently complex pSV-β-gal plasmid DNA under physiological conditions. For poly-**1** and poly-**3**, an N/P (ammonium positive charge on polymer/phosphate negative charge on DNA) ratio of 1.5 completely retarded the DNA; for poly-**2** this ratio was 2 (see Supporting Information). The difference in DNA/polymer binding efficiency is presumably due to the difference in the molecular weight of the polymer as poly-**2** has a slightly shorter chain length than poly-**1** and poly-**3**. Complex formation occurs largely because of entropic gains owing to the liberation of smaller counterions along the macromolecular chains.[19] Thus, as the chain is shortened (as for poly-**2**), there is less entropic gain during DNA complexation, which results in slightly weaker binding. The physical characteristics of the polymer/DNA complexes were then investigated by using AFM. Each polymer condensed DNA into spherical nanoparticles with typical diameters of 50–200 nm (Supporting Information), which is within the normal size range for cellular internalization.[20]

The transfection efficiency of the three hybrid polymers was tested and compared with PLL by using a luciferase-assay kit under serum-free conditions. As PLL and poly-**1**, poly-**2**, and poly-**3** have only primary amines and lack other amino residues to afford proton sponge effects, chloroquin (which is known to disrupt the membrane of the endosome) was used in all gene-transfection studies to enhance the endosomal release after entrance into the cell. Figure 4 summarizes the gene-transfection efficiency (normalized to the total cellular protein). Poly-**2** and poly-**3** showed a significantly higher transfection ability than PLL at similar N/P ratios. This is due primarily to the high toxicity of PLL at those concentrations. The lower transfection efficiency of poly-**1** compared to poly-**2** or poly-**3** is presumably due to lower local charge density on poly-**1** and the varied nature of the amino groups on the polymer chain. Poly-**2** and poly-**3** have both α-amino and more-flexible ε-amino functionalities, whereas poly-**1** has only α-amine groups. It has been reported that very subtle changes in polymer structure can result in significant changes in gene–transfection efficiency.[16a] Further structure–property correlation will be investigated in the future, which will provide information for structural optimization to improve the transfection efficiency.

In summary, we have described our concept for the design of saccharide–peptide hybrid copolymers as a new class of biomaterials. As examples, galactaro–dilysine (poly-**1**), trilysine (poly-**2**), and tetralysine(poly-**3**) hybrid copolymers were synthesized through interfacial polymerization of a galactose-derived monomer and corresponding L-lysine-derived peptide monomers. Enzymatic degradation, MTT tests, and immunological assays show that the hybrid copolymers are biodegradable, nontoxic, and nonimmunogenic. The hybrid copolymers were tested as vectors for possible application in gene delivery. EMSA, AFM, and luciferase-transfection studies demonstrate that the hybrid copolymers can efficiently compact plasmid DNA into soluble nanoparticles and be used as safe gene carriers. Given the natural abundance and functional diversity of saccharides and amino acids, their biodegradability, low cytotoxicity, and nonimmunogenicity, a diverse family of saccharide–peptide hybrid polymers are currently under development in our laboratory for various biomedical applications including gene/drug delivery and tissue engineering.

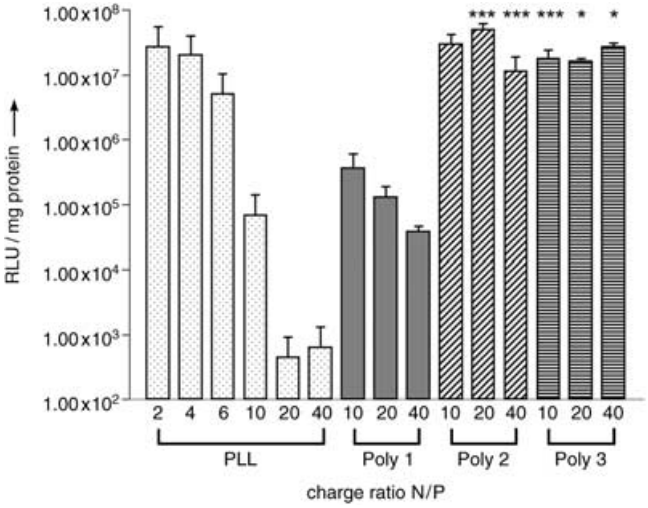


Figure 4. Luciferase gene-transfection data for the hybrid copolymers with PLL as control. Standard deviations are shown by the error bars ($n=3$). Relative light units (RLU) were normalized by using the total cellular protein in each well. The symbols * and *** indicate statistical significance at levels of $p<0.05$ and $p<0.001$, respectively, for the experimental polymers and PLL at corresponding N/P ratios. The p values were obtained by using Student—Newman–Keuls multiple comparisons testing.

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