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Turning peptides in comb silicone polymers^S

Said Jebors,^{a,b} Coline Pinese,^a Benjamin Nottelet,^a Karine Parra,^a Muriel Amblard,^a Ahmad Mehdi,^b Jean Martinez^{a†} and Gilles Subra^{a*‡}

We have recently reported on a new class of silicone-peptide^S biopolymers obtained by polymerization of di-functionalized chlorodimethylsilyl hybrid peptides. Herein, we describe a related strategy based on dichloromethylsilane-derived peptides, which yield novel polymers with a polysiloxane backbone, comparable with a silicone-bearing pendent peptide chains. Interestingly, polymerization is chemoselective toward amino acids side-chains and proceeds in a single step in very mild conditions (neutral pH, water, and room temperature). As potential application, a cationic sequence was polymerized and used for antibacterial coating.

Keywords: hydroxysilyl peptides; peptide materials; silicone; comb-polymers; polysiloxane

Introduction

Polysiloxanes, well known as ‘silicones’, are biocompatible, bio-inert, and biodurable materials extensively used since the 1960s in medicine [1] especially as medical device components for the fabrication of catheters, drains, shunts, orthopedics, and implants and also as injectable fluids for reconstructive surgery [2]. Despite their outstanding properties such as resistance to temperature and chemicals, high flexibility and elasticity, there is still a need for functional silicones whose properties can be adjusted by the addition of a bioorganic moiety. Sugar-silicon [3] or poly(L-lactic acid)-silicon hybrids [4] have been reported for cell recognition and bone tissue regeneration [5]. The association of silicone and peptides has also been proposed for improved cell-material interactions or detection purposes, but it has been mostly limited to functionalize silicone surface with short peptides [6,7]. To date, only few works report on the direct synthesis of peptide-silicon hybrids by a bottom-up approach. To the best of our knowledge, the only published study deals with silicones incorporating random homopolypeptide sequences obtained by ring opening polymerization of amino acids NCAs initiated by a poly(dimethylsiloxane) [8].

We recently reported on a strategy yielding siloxane polymers of well-defined peptide sequences starting from hybrid dimethylhydroxysilane-derivatized peptides [9]. In contrast to linear peptide polymers, which were obtained via N-terminal and C-terminal modifications, comb-peptide polymers were synthesized by addition of a lysine residue at the N-terminus, which was derivatized at its α and ε primary amino groups by isocyanopropyl dimethylchlorosilane. We suggest here a simple way to obtain comb-polymers of peptide sequences using methyl-dihydroxysilyl derivatives (Figure 1).

Material and Methods

Reagents

All solvents and reagents were used as supplied. Solvents used for Liquid Chromatography/Mass Spectrometry (LC/MS) were of

HPLC grade. Fmoc-amino acid derivatives, HBTU, and Fmoc-Rink amide ChemMatrix resin were purchased from Iris Biotech (Marktredwitz, Germany). DIEA and DMF, were obtained from Sigma-Aldrich (St. Louis, MO, USA). ICMDCS was obtained from Sikemia (Montpellier, France).

Synthesis

Example of solid phase peptide synthesis

The synthesis of H-Arg-Arg-Leu-Leu-Leu-NH₂ was performed by Fmoc/tBu SPPS [10]. H-Rink amide ChemMatrix Resin 0.49 mmol/g was swollen for 15 min in DCM. Fmoc deprotection was carried out with 20% piperidine in DMF for 20 min. Beads were washed with DMF (three times). About 2.5 ml of a 0.2-M solution of Fmoc-amino acid in DMF (5 eq.), 1 ml of a 0.5-M solution of HBTU (5 eq.) in DMF, and 0.5 ml of a 2-M solution of DIEA in DMF (10 eq.) were successively added to the resin. The coupling was carried out for 2 h. The resin was washed three times with DMF and once with DCM. After stepwise assembly of the peptide, it was cleaved from the resin with neat TFA for 1 h. After filtration, the solution was concentrated under reduced pressure. The peptide was precipitated with diethyl ether, filtered and dried under high vacuum, and

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Abbreviations: ICPMDCS, isocyanatopropyl methyl-dichlorosilane.

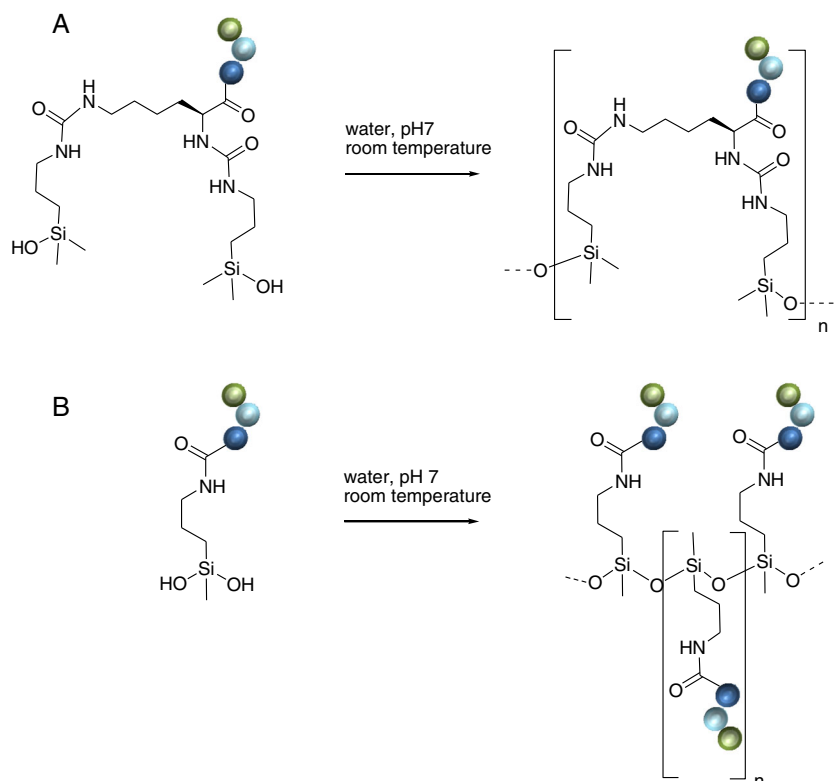


Figure 1. Comb-polymers from peptide sequences. (A) using N-terminally modified lysine with dimethyl hydroxysilyl groups [9]. (B) New strategy involving methyl dihydroxysilyl N-terminal peptides.

characterized by LC/MS. Procedures for the synthesis of other peptides are described in the Supporting Information Section.

Synthesis of silylated monomers 1–5

Commercially available 3-isocyanatopropyl dichloromethylsilane is used for the introduction of the chlorodimethylsilyl functionality onto peptides via their N-terminus.

Briefly, to the solution of peptide (0.5 mmol) in 500 μ l of DMF were added DIEA (6 eq.) and 3-isocyanatopropyl dichloromethylsilane (1.25 eq.). The reaction mixture was allowed to stir for 2 h at room temperature. After 120 min, the reaction was monitored by HPLC. Ether (50 ml) was poured into the reaction mixture to cause precipitation. The precipitate was suspended in ether and recollected. This procedure was repeated three times to remove 3-isocyanatopropyl dichloromethylsilane and DIEA. In the case of hybrid peptide **2**, an aliquot was deprotected in TFA/H₂O/TIS (92.5/5/2.5, v/v/v) solution to yield hybrid peptide **3**, which was precipitated by addition of diethylether (Supporting Information). All crude compounds were analyzed by analytical HPLC and LC/MS. It is worth noting that dichlorosilyl derivatives were hydrolysed during workup (or LC/MS analysis), yielding dihydroxysilyl hybrid peptides.

Polymerization

Hybrid peptide blocks **1–5** generated poly[methyl(peptido)siloxane] **P1–P5** through condensation mechanism between two SiOH groups with the release of water. Hybrid blocks were solubilized in a 1/1000 v/v TFA/water solution (0.5 g/ml). Phosphate buffer (pH 7.4) was added dropwise under stirring to slowly increase the pH of the reaction mixture above 7. Condensation occurred during buffer addition and induced precipitation of the polymer. The medium

was freeze dried, and polymer was obtained as a white powder or a sticky gel depending on the peptide sequence. The resulting polymers were insoluble in most solvents (i.e. DCM, MeOH, THF, EtOH, and chloroform), whereas **P1**, **P2**, and **P5** were soluble in DMF.

General Methods

LC/MS analyses

All compounds were analyzed on a Quattromicro (Micromass, Manchester, UK) triple-quadrupole mass spectrometer fitted with an electrospray interface. Chromatography was performed with a C18 reversed-phase column (VWR chromolith column, 50 mm \times 3.9 mm): 100% A to 100% B linear gradient over 5 min at a flow rate of 3 ml/min (A: 0.1% TFA in H₂O; B: 0.1% TFA in CH₃CN). Detection was performed at 214 nm. Positive ion electrospray mass spectra were acquired at a solvent flow rate of 204 μ l/min. Nitrogen was used for both the nebulizing gas and the drying gas. Data were obtained in a scan mode ranging from 100 to 1000 m/z in 0.1 s intervals; 10 scans were summed up to obtain the final spectrum.

Steric exclusion chromatography

Molecular weight of the polymers was determined by size exclusion chromatography (SEC) using (Waters corporation, USA) equipment fitted with a 5- μ m mixed C PLgel, 5- μ m MIXED-D (300 \times 7.5 mm) (Agilent Technologies, USA), and SHODEX RI 101 refractometric detector (Shodex, China). The mobile phase was DMF at 0.8 ml/min flow. Typically, the polymer (10 mg) was dissolved in DMF (2 ml), and the resulting solution was filtered through a 0.45- μ m Millipore filter before injection of 100 μ l of a filtered solution. Mn and Mw were expressed according to calibration using polystyrene standards.

Cytotoxicity and Antibacterial Assays

Cytotoxicity

Polymers were uniformly spread at the bottom of culture's well. Cytotoxicity was evaluated by lactate dehydrogenase (LDH) release measurement. LDH leakage into the culture medium as a result of fibroblasts L292 membrane lysis was evaluated with a cytotoxicity detection kit (LDH cytotoxicity Kit II, PromoKine, Heidelberg, Germany) according to the manufacturer's protocol. The LDH activity was determined spectrophotometrically after 30-min incubation at 25 °C of 10- μ l culture medium and 100- μ l reaction mixture, by measuring the oxidation of NADH at 450 nm in the presence of pyruvate, according to the manufacturer's kit instructions.

Antibacterial activity

Antibacterial activity of polymers was investigated according to the standard ISO 22196 with minor modifications.

Polymers were uniformly spread on glass strips. About 20 μ l of a 10^5 UFC ml⁻¹ bacterial suspension was dropped on glass strip-coated polymers. Coverslips were dropped off into the suspension in order to spread it, and samples were incubated at 37 °C. After 24 h, bacteria were detached from supports by washing with 10 ml of sterile saline solution. Samples were vortexed vigorously for 30 s and sonicated for 3 min. The viable cells in solution were then serially diluted, and 100- μ l aliquots were spread on gelose tripticase soja plates for CFU determination.

| Table 1. Hybrid peptides (HO) ₂ SiMe-(CH ₂) ₃ NHCO-peptide and comb-peptide polysiloxanes [OSiMe-(CH ₂) ₃ NHCO-peptide] _n | | | | | | | | |
|---|---|------------------------|--------------------------|---------|-------|-----------------|--------------------------------|----------------|
| Monomer | | | | Polymer | | | | |
| Entry | Sequence R=(HO) ₂ SiMe-(CH ₂) ₃ NHCO- | M _w (calc.) | m/z [M + H] ⁺ | Polymer | Tg °C | Mn ^a | DP _{SEC} ^a | Đ ^a |
| 1 | R-Phe-Phe-OH | 473.6 | 474.1 | P1 | No Tg | 11 609 | 25 | 1.28 |
| 2 | R-Ahx-Arg(Pbf)-Arg(Pbf)-NH ₂ | 1108.4 | 1108.6 | P2 | No Tg | 16 950 | 15.4 | 1.08 |
| 3 | R-Ahx-Arg-Arg-NH ₂ | 603.8 | 604.3 | P3 | No Tg | Insoluble | — | — |
| 4 | R-Leu-Leu-Leu-Arg-Arg-NH ₂ | 830.1 | 830.7 | P4 | No Tg | Insoluble | — | — |
| 5 | c[Asp-DPhe-Lys(R)-Arg-Gly] | 764.9 | 765.4 | P5 | No Tg | 8993 | 12 | 1.25 |

^aDispersity Đ and the average molecular weight Mn (g mol⁻¹) were estimated by steric exclusion chromatography (SEC) in DMF, using polystyrene as standard. Mn allows the calculation of degree of polymerization (DP).

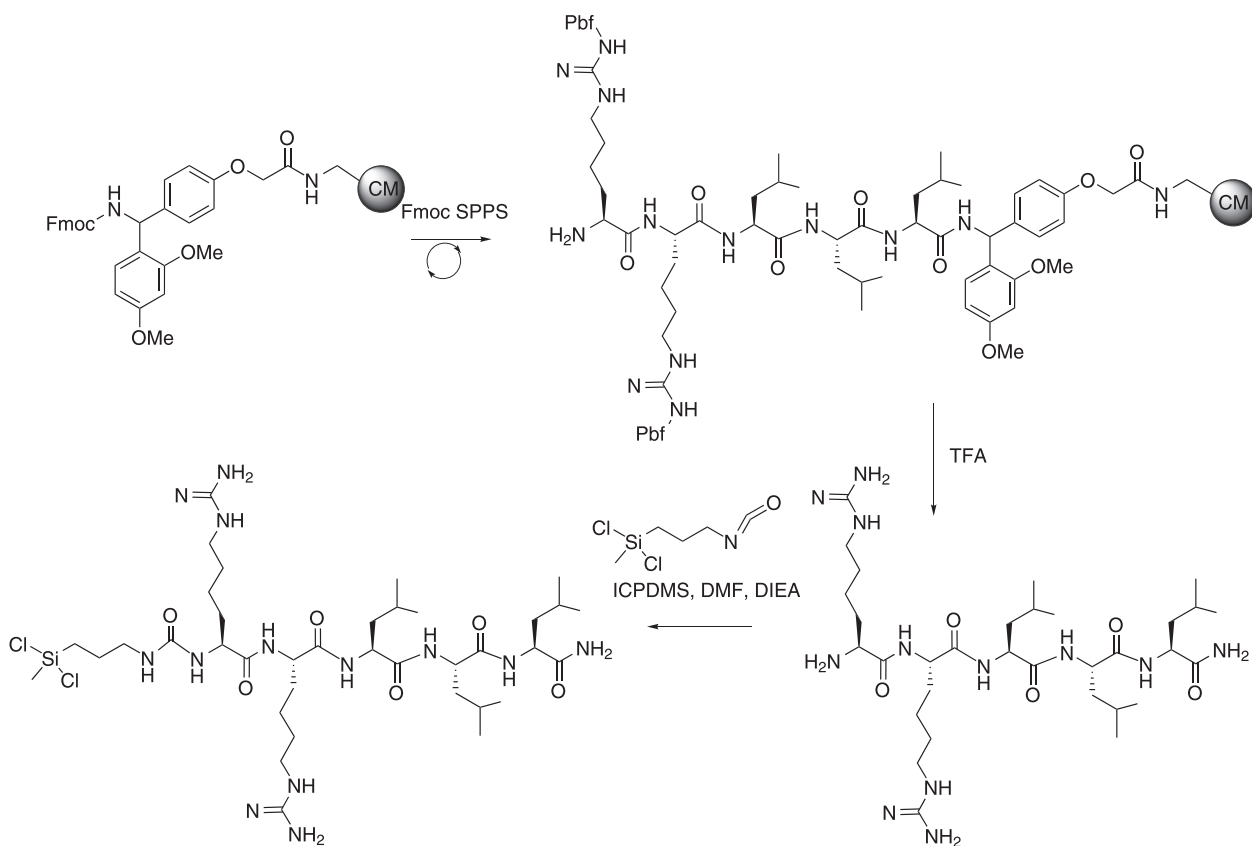


Figure 2. Synthesis of dichloromethylsilyl hybrid peptide 4.

Result and Discussion

Five hybrid peptides were prepared (Table 1). Hybrid dipeptide **1** was first used as a model sequence to demonstrate the feasibility of the polymerization. Sequences **3** and **4** were selected as small cationic peptides containing arginine residues, which could yield polymers able to interact and potentially disrupt bacterial membranes. Noteworthy, a silylated analog of the Ahx-Arg-Arg-NH₂ tripeptide was already used in our group to graft a glass surface, providing interesting antibacterial properties [11]. Hybrid peptide **2** was the Pbf-protected analog of **3**. Sequence **5** is the cyclic peptide RGDfK derivative, a ligand of integrin receptors, which has been used to improve cell adhesion to surfaces [12–14]. After the synthesis of the peptides sequences either in solution or on solid support (Figure 2) and subsequent silylation using ICPMDCS, methylchlorosilyl hybrid peptides were obtained and solubilized in DMF. Polymerization of hydrolyzed methylchlorosilyl hybrid peptides started by dropwise addition of a pH 7.4 buffer at room temperature. Condensation of hydroxysilane groups proceeded quickly, and the polysiloxane-peptide precipitated within 2 h without use of any chemical reagent. Polymers were analyzed by SEC to determine the average molecular weight (Mn) according to calibration using polystyrene standards. Glass transition temperatures (T_g) were determined by differential scanning calorimetry analyses, which were performed from -150 °C to 110 °C (Table 1). In contrast to polydimethylsiloxanes, all tested polymers exhibited no T_gs in

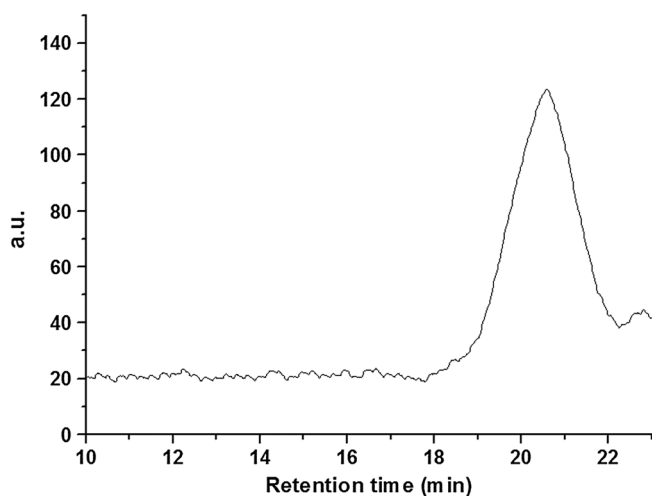


Figure 3. SEC chromatogram (DMF, LiBr 1%) of polymer **P2** (UV detection at 254 nm).

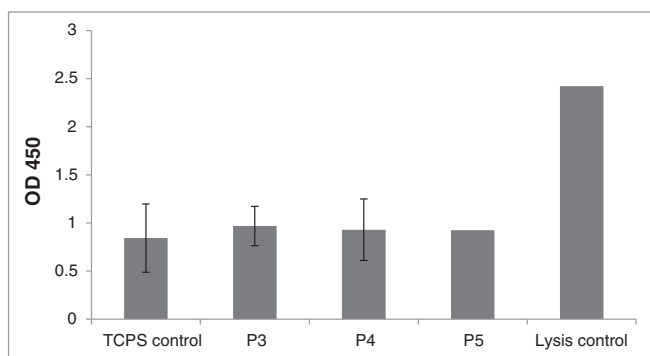


Figure 4. LDH level for L929 fibroblasts in contact with polymers **P3**, **P4**, and **P5**.

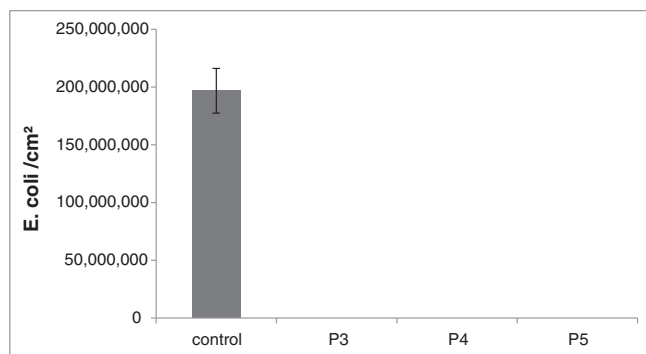


Figure 5. *Escherichia coli* number per cm² after 24 h of contact with polymers.

this range. This could be a consequence of hyperbranched and hybrid structure of the comb-peptide polymers. SEC chromatograms showed **P1** had a significant molecular weight of ≈ 11600 corresponding to an average polymerization degree of 25, whereas **P2** Mn (**P2**) ≈ 16950 , and **P5** Mn(**P5**) $\approx 8993 \text{ gmol}^{-1}$ (Table 1), corresponded, respectively, to polymerization degrees of ca. 15.4 and 12. This difference could be attributed to the structure of monomers **2** and **5** composed of more bulky organics parts, which could slow down the polymerization process. Dispersity \bar{D} was found to be close to 1, demonstrating that, for a given polymer, chain lengths are similar, see Figure 3

Amorphous polymers **P3**, **P4**, and **P5** were dropped off *without solvent* and spread as a layer on a glass surface previously activated by piranha treatment (H₂SO₄/H₂O₂ 4/1 v/v). They were incubated overnight at 40 °C in order to immobilize the polymer on the glass surface.

Cytotoxicity was evaluated by LDH release measurement. After 24 h contact with polymers, fibroblasts (Figure 4) showed a low level of LDH (OD₄₅₀ = 0.92) close to fibroblasts level on tissue culture polystyrene (TCPS) control (OD₄₅₀ = 0.84). In comparison, lysed fibroblasts exhibited a high LDH level (OD₄₅₀ = 2.42). This first result shows that polymers are non-cytotoxic and can be used in a biomedical application, alone or, interestingly, in combination with polydimethyl siloxane units.

As a proof of concept, we evaluated our polymers for their antibacterial effects against *Escherichia coli*. Indeed, in most cases, infections are initiated by the adherence of planktonic bacterial cells onto the biomaterial surface. Silicone medical devices such as catheters, laryngeal masks, or septums may be prompted to progressive colonization and biofilm formation. It is therefore important for potential biomedical application and further development that comb-cationic polymers prepared by our methodology possess bactericidal properties. Thus, we selected the three polymers (**P3**, **P4**, and **P5**) that display guanidine groups in their sequences.

As shown in Figure 5, no bacteria were observed after 24-h culture on polymers **P3–P5**. The antimicrobial effect results in an eight-log reduction of living bacteria upon incubation. Surprisingly, no significant difference of activity was observed between **P3**, **P4** (Arg-Arg derivatives), and **P5** (cRGDfK). All of them exhibit significant antimicrobial properties toward *E. coli*.

Conclusion

In this study, we propose a new class of bioorganic polysiloxanes obtained by oligomerization of well-defined hybrid peptide sequences bearing a dimethylchlorosilane moiety. Straightforward,

hydrolytic condensation occurs in water at room temperature and does not require any catalyst or chemical reagent. As a proof of concept, we prepared comb-polymers displaying cationic peptides, which proved to be efficient against *E. coli*. We believe that this type of polymerization in water can easily be scaled up and can find several applications to advantageously add dedicated biological properties lacking to conventional silicones.

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Supporting Information

Additional supporting information may be found in the online version at the publisher's web site.