Zwitterionic Polymer Conjugated Glucagon-like Peptide-1 for Prolonged Glycemic Control

Caroline Tsao, Peng Zhang, Zhefan Yuan, Dianyu Dong, Kan Wu, Liqian Niu, Patrick McMullen, Sijin Luozhong, Hsiang-Chieh Hung, Yu-Hong Cheng, and Shaoyi Jiang*

ABSTRACT: Glucagon-like peptide-1 (GLP-1) is of particular interest for treating type 2 diabetes mellitus (T2DM), as it induces insulin secretion in a glucose-dependent fashion and has the potential to facilitate weight control. However, native GLP-1 is a short incretin peptide that is susceptible to fast proteolytic inactivation and rapid clearance from the circulation. Various GLP-1 analogs and bioconjugation of GLP-1 analogs have been developed to counter these issues, but these modifications are frequently accompanied by the sacrifice of potency and the induction of immunogenicity. Here, we demonstrated that with the conjugation of a zwitterionic polymer, poly(carboxybetaine) (pCB), the pharmacokinetic properties of native GLP-1 were greatly enhanced without serious negative effects on its potency and secondary structure. The pCB conjugated GLP-1 further provided glycemic control for up to 6 days in a mouse study. These results illustrate that the conjugation of pCB could realize the potential of using native GLP-1 for prolonged glycemic control in treating T2DM.

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a long-term progressive metabolic disease characterized by high blood glucose (hyperglycemia) due to the dysfunction of pancreatic β-cells and insulin resistance.1,2 The incidence of diabetes is expected to increase as it has affected 463 million adults (20–79 years old) worldwide in 2019, in which T2DM is accounting for ~90% of all cases.3 The number of people affected by diabetes is expected to reach 700 million by 2045, estimated by the International Diabetes Federation.4 Native glucagon-like peptide-1 (GLP-1 (7–36), abbreviated to GLP-1) is an incretin peptide secreted from intestinal L-cells in response to meal intake, in order to induce insulin secretion from pancreatic β-cells and suppress glucagon release in a glucose-dependent fashion.4–6 While overweight and obesity are now considered as the major risk factor of non-insulin-dependent diabetes mellitus, continuous subcutaneous infusion of GLP-1 has resulted in a sustained weight loss over a period of at least 6 weeks in a human study.7 Therefore, GLP-1 shows great potential use in the treatment of T2DM and obesity. However, native GLP-1 has limitations for clinical use due to the rapid proteolytic inactivation and degradation by dipeptidyl peptidase IV (DPP-IV) enzyme.8–10

The peptide bond in Ala8-Glu9 is cleaved by DDP-IV which results in a metabolite of GLP-1(9–36) that has a 100-fold lower binding affinity compared to the native intact peptide11,12. To overcome this shortcoming, two possible approaches have been considered: (1) utilize natural analogs or synthetic analogs by modifying the residues vulnerable to DPP-IV, and (2) “shield” the native peptide with synthetic materials to protect it from proteolytic inactivation.

A number of GLP-1 analogs, mainly GLP-1 receptor agonists (GLP-1 RAs), have been developed based on the first approach, including exenatide, a lizard-derived analog; taspoglutide, an analog with α-aminoisobutyric acid (Aib) substitutions; and liraglutide, an acylated derivative.13–15 Unfortunately, these GLP-1 analogs have raised undesired immune responses: antidrug antibodies (ADA) toward GLP-1 RAs due to the foreignness introduced upon residue modifications. The incidences of antibody formation in clinical use were 45.4% of the patients treated with exenatide, 49% with tapsoglutide, 69.8% with lixisenatide, and 8.6% with liraglutide.16–18

The generation of ADA could lead to a compromise of therapeutic efficacy of GLP-1 RAs and an increase in hypersensitivity reactions after administration.19–22 Thus, the delivery of native GLP-1 for treating T2DM is still an attractive idea, as there is low risk of ADA generation, which is desirable for long-term treatments. On the other hand, limited studies have been done using the second approach to prevent rapid proteolytic inactivation. Therefore, we aim to further explore the possibility...
One common problem that GLP-1 and GLP-1 analogs face is the short circulation half-life from rapid renal clearance due to their low molecular weights. The conjugation of natural or synthetic materials has been shown to improve the pharmacokinetic (PK) properties of GLP-1 analogs. Conjugation of different lengths of poly(ethylene glycol) (PEG) or polysaccharides has increased the half-life from 1.5−5 min to 12.1−30.3 h when injected subcutaneously in rodents. Integration of human serum albumin or fragment crystallizable region (Fc) has improved the half-life to 8.5−38.2 h in rodents when injected subcutaneously. Lipidation of the GLP-1 analogs also extended the half-life to 12−46.1 h when delivered intravenously in rodents. Yet, the addition of materials could sacrifice the activities of the GLP-1 and GLP-1 analogs. The conjugation of polymers (such as PEG) or human serum albumin has been reported to dramatically reduce the potencies of GLP-1 analogs due to unwanted interactions between the bulk conjugate and the peptide, and steric hindrances of the bulk conjugate. Furthermore, some synthetic materials, such as PEG, have been demonstrated to be immunogenic and

Scheme 1. Through the Conjugation of Zwitterionic Poly(carboxybetaine) Polymer onto Native Glucagon-like Peptide-1, the Bioconjugate GLP1-pCB Has Three Potential Advantages: It (1) Maintains the Secondary Structure and Activity of GLP-1, (2) Enhances the Pharmacokinetic Properties In Vivo, and (3) Provides Prolong Glycemic Control in a Mouse Study

Figure 1. A. Synthetic scheme for pCB conjugation onto GLP-1. B. Nuclear magnetic resonance spectroscopy (1H NMR, 300.10 MHz, D2O) profile of each conjugation step. The appearance of the small peak indicates the successful addition of the maleimide, while the disappearance indicates the successful conjugation onto GLP-1. C. Gel permeation chromatography (GPC) traces of GLP1-pCB. The left-shift of GLP1-pCB peak of suggests the success of conjugation as GLP1-pCB is larger than pCB alone.

of using synthetic materials to improve the therapeutic effects of native GLP-1 in this study.

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vulnerable to pre-existing anti-PEG antibodies. Therefore, it is ideal to use synthetic alternatives that would not affect peptide activity and have low immunogenicity for peptide conjugation.

Zwitterionic polymer poly(carboxybetaine) (pCB) has been demonstrated to be efficacious in protecting biologics from undesired interactions in the biological environment and in improving the PK properties of the biologics. Due to their superhydrophilic nature, the conjugation of pCB polymers has been demonstrated to retain protein stability and bioactivity. Moreover, studies have shown that pCB polymers induce little to no anti-pCB antibodies even when conjugated to highly immunogenic proteins. In this study, we develop a bioconjugate of native GLP-1 with a superhydrophilic zwitterionic polymer pCB. We first investigate the secondary structure and activity of the native GLP-1 after the conjugation of pCB. The PK properties of the bioconjugate, and its ability to provide glycemic control, are next evaluated. We aim to demonstrate the potential of using pCB-conjugated native GLP-1 for prolonged glycemic control (Scheme 1).

**RESULTS AND DISCUSSION**

The structure–activity relationship of GLP-1 has been well investigated, and several amino acid residues on the N-terminus have been identified as critical for receptor binding and activation. Previous studies on the site-specific conjugation of PEG onto GLP-1 have demonstrated that the N-terminus of GLP-1 is essential for receptor activation. Though the conjugation of PEG close to the N-terminus can mitigate the proteolytic inactivation of GLP-1 by DDP-IV, the potency of GLP-1 was greatly reduced. Hence, in this study, we develop a bioconjugate of native GLP-1 with a superhydrophilic zwitterionic polymer pCB. We first investigate the secondary structure and activity of the native GLP-1 after the conjugation of pCB. The PK properties of the bioconjugate, and its ability to provide glycemic control, are next evaluated. We aim to demonstrate the potential of using pCB-conjugated native GLP-1 for prolonged glycemic control (Scheme 1).

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**Figure 2.** A. Circular dichroism (CD) spectroscopy of pCB, GLP-1, and GLP1-pCB. GLP1-pCB maintains the secondary structure of GLP-1. B. In vitro insulinotropic activity of GLP-1 and GLP1-pCB. Properties of both are summarized in Table 1. All studies were done in triplicate.
GLP-1 with a similar molecular weight of 50 kDa only retained an EC_{50} of 1870 nM, corresponding to 0.03% of the un conjugated peptide. Similarly, when GLP-1 was conjugated to a human serum albumin (66 kDa) through a short PEG linker, the potency of GLP-1 was reduced by 3 orders of magnitude. Even though there was a decrease in potency, E_{max} of GLP1-pCB was 98.8% with respect to GLP-1 set as 100%, suggesting that conjugation of pCB would not affect the activation of GLP-1 receptors, but only the binding affinity. We hypothesize that pCB draws water away from the relatively hydrophobic peptide, allowing the peptide and the receptor to interact by shifting the equilibrium, which maintains the potency and efficacy of GLP-1. Furthermore, as suggested before, it is hypothesized that the minimized nonspecific interactions between pCB and the GLP-1 receptor due to the superhydrophilic nature of the zwitterionic polymer can further facilitate the interaction between the peptide and its receptors.

The short circulation half-lives of GLP-1 and GLP-1 analogs due to their small size is a major challenge for clinical use. The area under the curve (AUC) of GLP-1 reported previously (7.24 ± 1.12 days) further illustrated the possibility of a single subcutaneous injection of GLP-1 being able to enter the systemic bloodstream and maintain a prolonged circulation profile. It has been reported that protein therapeutics and nanoparticles with a molecular weight more than 16 kDa administered subcutaneously exhibit limited direct transport into the blood capillaries upon administration and enter the systemic circulation via an indirect route, through the lymphatic system. This could possibly be due to the prevention of nonspecific interaction with the environment owing to the superhydrophilicity of pCB.

As GLP1-pCB showed good activity in vitro and improved circulation half-life in vivo, we last evaluated its pharmacodynamic (PD) profile through the intraperitoneal glucose-tolerance test (IPGTT) (Figure 4). C57BL/6 mice were subcutaneously injected with a single dose of 300 nmol/kg (equivalent to 1 mg/kg of GLP-1) of GLP1-pCB, or saline (negative control) at time 0. IPGTTs were performed once on each mouse at different time points: 6, 30, 54, 78, 102, 126, and 150 h after sample injection. Mice were fasted for 6 h before the IPGTT, when the mice were challenged with an intraperitoneal injection of glucose at 2 g/kg. Blood glucose levels were monitored for 120 min after the glucose challenge. The loss of activity after 150 h could be due to the low concentration of GLP1-pCB remaining in the bloodstream. The area under the curve (AUC_0−120) further illustrated the differences in the accumulative blood glucose level for up to 7 days (Figure 4I). These results showed the GLP1-pCB exerted activity for up to 6 days, suggesting that pCB could provide protection for GLP-1 from proteolytic cleavages in the bloodstream and provide prolonged glycemic control.

### CONCLUSIONS

In this study, we developed a bioconjugate GLP1-pCB consisting of the native GLP-1 peptide with a zwitterionic polymer pCB. We first demonstrated that the conjugation of pCB onto the peptide did not alter the secondary structure of GLP-1. Even though the GLP1-pCB conjugate had a modest loss of potency due to steric hindrance as expected for polymer–protein conjugates, it still possessed very good efficacy in vitro.

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Table 1. *In Vitro* Potency and Relative Efficacy of GLP-1 and GLP1-pCB

<table>
<thead>
<tr>
<th>GLP-1</th>
<th>Potency EC(_{50}) (nM)</th>
<th>Relative E(_{\text{max}}) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLP-1</td>
<td>1.08 ± 1.17</td>
<td>100%</td>
</tr>
<tr>
<td>GLP1-pCB</td>
<td>7.24 ± 1.12</td>
<td>98.8%</td>
</tr>
</tbody>
</table>

“Relative E\(_{\text{max}}\): Maximum insulin release relative to GLP-1 as 100.”

Table 2. Pharmacokinetics Profile of GLP1-pCB

<table>
<thead>
<tr>
<th>T(_{1/2}) (h)</th>
<th>T(_{\text{max}}) (h)</th>
<th>C(_{\text{max}}) (ng/mL)</th>
<th>AUC(_{0-\infty}) (ng/mL·h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>43.0</td>
<td>13.4</td>
<td>1427.1</td>
<td>72931.1</td>
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*ng/mL: the concentration of GLP1-pCB is expressed as the amount of GLP-1 detected in the serum. 10 ng/mL of GLP-1 detected is equivalent to 3 nmol/mL of GLP1-pCB in the serum.

(T\(_{1/2}\) = 12.1–38.2 h), while C\(_{\text{max}}\) was slightly better than other modifications of GLP-1 reported previously (C\(_{\text{max}}\) = 684–1022 ng/mL). These results indicated that more GLP1-pCB was able to enter the systemic bloodstream and maintain a prolonged circulation profile.

Figure 3. Circulation profile of a single subcutaneous injection of GLP1-pCB. The concentration of GLP1-pCB is expressed as the amount of fluorescent-labeled GLP-1 detected in the serum.
GLP1-pCB had a prolonged circulation half-life and exerted activity up to 6 days after a single subcutaneous injection in a mouse study. While we present proof-of-concept experiments, more studies regarding the long-term immunogenicity and the optimization of the GLP1-pCB construct should be done in the future. The conjugation of pCB provides a potential solution for the fast clearance and rapid inactivation of GLP-1, which shows promise for providing long-term glycemic control for treating T2DM.

Figure 4. Intraperitoneal glucose tolerance test (IPGTT) of GLP-1 and GLP1-pCB. A. Experimental design for the IPGTT. Mice were first subcutaneously injected with a single dose of 300 nmol/kg GLP-1, GLP1-pCB, or saline (labeled as Control) at time 0 h. IPGTTs were then conducted on animals with an intraperitoneal injection of glucose (2 g/kg) at different time points: 6, 30, 54, 78, 102, 126, and 150 h post-sample injection. Six animals were challenged with IPGTT for each time point. Blood glucose levels of each animals were then monitored for 120 min during each IPGTT. B.–H. Blood glucose profiles of IPGTT at different time points after sample injection. I. Accumulative blood glucose AUC$_{0-120}$ (area under the curve) profiles of IPGTT. The AUC was calculated based on the blood glucose profiles from B. to H. Results are reported as mean ± SD *p < 0.05, **p < 0.01.
**EXPERIMENTAL PROCEDURES**

**General Experimental Details.** Glucagon-like Peptide-1 (GLP-SH, HEGGTFTSDVSSYLEGQAAKEFIALWVGRSH) was purchased from GenScript (Piscataway, USA). Poly(carboxybetaine) (pCB) was synthesized as reported previously. In brief, 3-acrylamido-N-(2-(tert-butoxy)-2-oxoethyl)-N,N-dimethylpropan-1-aminium (t-Butyl CBAAm) and chain transfer agent (CTA) were synthesized. pCB polymer was next prepared by a combination of reversible addition–fragmentation chain transfer (RAFT) polymerization, aminoxylation, acid deprotection, and amine-to-thiol conversion steps utilizing the t-Butyl CBAAm and CTA prepared earlier. N,N-Maleimidopropyl-oxysuccinimide ester (BMPS) was from TCI America. Amicon Ultra centrifugal filter units were purchased from EMD Millipore (Billerica, MA). Pierce 660 nm Protein Assay was purchased from Thermo Fisher Scientific (Waltham, MA). Fetal bovine serum (FBS), Gibco RPMI 1640 (ATCC modification) medium, and RPMI 1640 medium (none ATCC modification) were all purchased from Thermo Fisher Scientific (Waltham, MA). RIN-mSF cells were purchased from ATCC (Manassas, VA). Alexa Fluor 555 NHS Ester (succinimidyl ester) was purchased from Thermo Fisher Scientific (Waltham, MA).

**Preparation of pCB-Conjugated GLP-1 (GLP1-pCB).** pCB (72 mg) were first dissolved in phosphate-buffered saline (PBS) pH 7.6 at 36 mg/mL, slowly mixed with 7.23 μM BMPS/DMSO (100 μL of 20 mg/mL), and reacted for 40 min at room temperature. The reaction mixture was then purified through centrifugal filters with a molecular cutoff at 10 kDa through multiple washes using PBS pH 7.35. GLP-1 (5 mg) was first dissolved in deionized water at 12.5 mg/mL (400 μL). The GLP-1 solution was then mixed with maleimide-modified pCB at 1.725 mg/mL (400 μL + 2500 μL) in PBS pH 7.35 by slowly dripping droplets while stirring, and reacted for 2 h at room temperature. Reaction mixture was then transferred to 4 °C fridge and reacted overnight. Reaction mixture was then purified and collected through the ENrich Size Exclusion Chromatography 650 column (10 mm × 300 mm), using NGC 10 Quest Chromatography System (Biorad). The concentration of GLP1-pCB was determined through Pierce 660 nm Protein Assay. Products from each step of the conjugation procedure were determined using 1H NMR (300.10 MHz, D2O) and the Agilent Technologies 1260 Infinity binary high performance liquid chromatography (HPLC) system with Waters UltraHydrogel 1000 column (7.8 mm × 300 mm).

**Structural Analysis of GLP1-pCB.** GLP1-pCB and GLP-1 were characterized by circular dichroism. A Jasco J-720 spectropolarimeter was used to measure the far-UV spectra of the proteins diluted to a concentration range of 20–200 μM in 20 mM sodium phosphate buffer, pH 7. The mean residue ellipticity was measured from 190 to 250 nm in a 0.1 cm path length quartz cuvette at 25 °C. All spectra were accumulated with standard sensitivity. Studies were done in triplicate to ensure reproducibility.

**In Vitro Insulinotropic Activity Assay.** The insulinotropic activity of GLP-1 and GLP1-pCB was evaluated by static incubation of RIN-mSF cells. Cells were seeded in 10% FBS/Gibco RPMI 1640 (ATCC modification) medium in Corning Costar Flat Bottom cell culture plate, 96 well, at a density of 1.5 × 104 cells/well, and grown overnight at 37 °C, under 5% CO2. Acute tests for insulin release upon contact with samples were preceded by 2 h preincubation at 37 °C, under 5% CO2 in glucose-free-serum-free RPMI 1640 medium (no ATCC modification). Test incubation was performed in the presence of 2 mM glucose (Thermo Fisher Scientific) and samples in serum-free RPMI 1640 medium at the final concentration from 10−12 to 10−6 M. After 30 min of incubation, the supernatant of each well was collected and centrifuged at 1000 × g for 5 min at 10 °C. The insulin content was analyzed forthwith by using rat insulin ELISA kit (Thermo Fisher Scientific). Concentration–response curves were analyzed by using a nonlinear curve fitting computer program (GraphPad Prism, San Diego, CA, USA), which yielded EC50 (concentration producing half-maximal response) and Emax (maximal effect) values.

**Pharmacokinetic Studies.** All animal experiments in this study adhered to federal guidelines and were approved by the University of Washington Institutional Animal Care and Use Committee. GLP1-pCB was first tagged with Alexa Fluor 555 with NHS ester targeting the lysines (K) of the construct. GLP1-pCB was dissolved in 1 mL of PBS pH 7.6 at 2 mg/mL; Alexa Fluor 555 was dissolved in 100 μL DMSO at 20 mg/mL. The Alexa Fluor 555/DMSO mixture was then slowly mixed with the GLP1-pCB solution by dripping droplets while stirring and reacted for 2 h at room temperature. The reaction mixture was then transferred to 4 °C refrigeration and reacted overnight. Reaction mixture was then purified and collected through multiple buffer exchanges (PBS pH 7.4) using 30 kDa MWCO Amicon Ultra centrifugal filter units.

Male C57BL/6 mice (5–8 weeks old, n = 12) received subcutaneous injections of 1 mg/kg Alexa Fluor 555-labeled GLP1-pCB conjugate at time 0 h. Blood samples were collected from tail vein at 6, 30, 54, 78, 102, 126, and 150 h post-administration. Serum samples were prepared from the blood samples by centrifuging at 11,000 rpm for 10 min. The concentrations of GLP1-pCB were determined by the fluorescence intensity (excitation at 552 nm, emission at 576 nm) measured with a microplate reader (BioTek, Cytation 5). Pharmacokinetic parameters were determined through PKSolver.56

**Pharmacodynamic Studies with Intraportaline Glucose-Tolerance Test (IPGGT).** Male C57BL/6 mice (5–8 weeks old, n = 144) received subcutaneous injections of pharmaceutical-grade saline, 1 mg/kg of GLP-1, or 1 mg/kg of GLP1-pCB conjugate at time 0 h. At different time points, including 6, 30, 54, 78, 102, 126, and 150 h, 6 animals were challenged with IPGGT. Each animal has only been challenged once throughout the whole experiment. For each IPGT challenge, animals were first fasted for 6 h. After the fast, they were given an intraperitoneal injection of sterilized pharmaceutical-grade glucose at 2 g/kg. Blood glucose levels of the 6 animals at each IPGGT time point were measured at time 0, 20, 40, 60, 80, 100, and 120 min after the glucose challenge with a self-monitoring blood glucose meter (Advocate, Redi-Code+, 2–5 μL of blood samples). The blood glucose levels were plotted over time, and the AUC0−120 was obtained using GraphPad Prism7. No animals were excluded due to complications.

**Statistical Analysis.** Results are reported as mean ± SD. Two-tailed Student’s t-test was used to compare two small sets of quantitative data.
AUTHOR INFORMATION

Corresponding Author
Shaoyi Jiang — Department of Chemical Engineering, University of Washington, Seattle, Washington 98195, United States; orcid.org/0000-0001-3986-6899; Email: sjiang@uw.edu

Authors
Caroline Tsao — Department of Chemical Engineering, University of Washington, Seattle, Washington 98195, United States; orcid.org/0000-0001-5815-4035
Peng Zhang — Department of Chemical Engineering, University of Washington, Seattle, Washington 98195, United States
Zhefan Yuan — Department of Chemical Engineering, University of Washington, Seattle, Washington 98195, United States; orcid.org/0000-0002-4724-6214
Dianyu Dong — Department of Chemical Engineering, University of Washington, Seattle, Washington 98195, United States; School of Chemical Engineering and Technology and Key Laboratory of Systems Bioengineering of Ministry of Education, Tianjin University, Tianjin 300350, China; orcid.org/0000-0002-2293-7959
Kan Wu — Department of Chemical Engineering, University of Washington, Seattle, Washington 98195, United States; orcid.org/0000-0002-8900-5574
Liqian Niu — Department of Chemical Engineering, University of Washington, Seattle, Washington 98195, United States
Patrick McMullen — Department of Chemical Engineering, University of Washington, Seattle, Washington 98195, United States
Sijin Luo zhong — Department of Chemical Engineering, University of Washington, Seattle, Washington 98195, United States
Hsiang-Chieh Hung — Department of Chemical Engineering, University of Washington, Seattle, Washington 98195, United States
Yu-Hong Cheng — Department of Chemical Engineering, University of Washington, Seattle, Washington 98195, United States

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.bioconjchem.0c00286

Notes
The authors declare no competing financial interest.

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ABBREVIATIONS

GLP-1, glucagon-like peptide-1; T2DM, type 2 diabetes mellitus; pCB, poly(carboxybetaine); DPP-IV, dipeptidyl peptidase IV; GLP-RAs, GLP-1 receptor agonists; Aib, α-aminoisobutyric acid; AD, antidrug antibodies; PK, pharmacokinetics; PEG, poly(ethylene glycol); Fc, fragment crystallizable region; 1H NMR, nuclear magnetic resonance spectroscopy; \( E_{\text{max}} \), maximum response (insulin release); \( EC_{50} \), half-maximal response; BMP5, N-β-maleimidopropyl-oxysuccinimide ester linker; GPC, gel permeation chromatography; GLP1-pCB, poly(carboxybetaine) polymer conjugated glucagon-like peptide-1; \( T_{1/2} \), circulation half-life; \( C_{\text{max}} \), maximum serum concentration observed; PD, pharmacodynamic; IPGT, intraperitoneal glucose-tolerance test; AUC\(_{0-120} \), area under the curve (from 0 to 120 min); HPLC, high performance liquid chromatography.

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