

Tethered DNA hairpins facilitate electrochemical detection of DNA ligation†

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A novel electrochemical assay for DNA ligase activity is described. The assay exploits the properties of DNA hairpins tethered at one terminus to a gold electrode and labelled at the other with a ferrocene group for rapid characterisation of DNA status by cyclic voltammetry. Successful ligation of 'nicked' DNA hairpins is indicated by retention of the ferrocene couple when exposure to DNA ligase is followed by conditions that denature the hairpin. The results demonstrate the simplicity of integrating electrochemical detection with hairpin based biosensors and illustrate a new approach to the assay of DNA ligases, of which the NAD⁺-dependent enzymes represent a potential broad spectrum antibacterial drug target.

Introduction

The ability to produce nucleic acids of defined sequence by chemical synthesis has revolutionised analysis in the biological, pharmaceutical and forensic arenas. Synthetic oligonucleotides have become essential elements of methods designed for sequence specific DNA detection and the characterisation of DNA interactions with proteins, drugs and other chemicals. The selectivity and specificity of these approaches is in large part due to the inherent chemical properties of DNA. Many of these properties are evident in the operation of 'molecular beacons', pre-eminent amongst DNA containing biosensors, which exploit DNA hybridisation chemistry.^{1,2} The beacons are DNA hairpins terminated at opposite ends by a fluorophore and quencher; the presence of a complementary DNA sequence opens the hairpin, separates the fluorophore and quencher and a signal is generated.

The majority of assays exploiting DNA hairpins have been developed for homogeneous detection of complementary DNA sequence through changes in fluorescence intensity. However, opportunities to exploit hairpins for the detection of a wider range of analytical targets with alternate methods of signal transduction have begun to be realised. Hairpins have been designed that report quantitatively on the activity of DNA processing enzymes.^{2,3} Heterogeneous assays in which hairpins are tethered to surfaces that form an *integral* part of the signal transduction method are also known.⁴ An elegant example of this approach utilises redox-labelled hairpins tethered to gold electrodes for reagentless, sequence-specific DNA detection through electrochemical analysis.⁵ Here we adopt this approach to extend the analytical target for a tethered hairpin based sensor with proof of concept for a novel electrochemical assay for a DNA processing enzyme, DNA ligase.

DNA ligases seal breaks, or 'nicks', in the backbone of duplex DNA and are essential to all organisms.^{6,7} The

different cofactor specificity of bacterial and human DNA ligases (NAD⁺ vs. ATP) has suggested the former as a promising target for broad-spectrum antibacterial compounds and has stimulated the development of assays alternate to the traditional electrophoretic approaches.^{8–10} Our assay employs a nicked DNA hairpin as the ligase substrate, Fig. 1. The hairpin is tethered to a gold electrode through a terminal thiolate. A ferrocene label at the remote terminus provides a redox reporter for rapid characterisation of DNA status by cyclic voltammetry.^{5,11–13} Successful ligation of the DNA substrate is indicated by retention of the ferrocene couple after incubation with DNA ligase is followed by exposure to conditions that denature the hairpin.

Experimental protocols

Materials

Expression and purification of the NAD⁺-dependent DNA ligase, LigA, from *Escherichia coli* was as described previously.^{14,15} Oligonucleotides A–J (Table 1) were supplied by MWG Biotech or SIGMA-Genosys. All other reagents were of Analar quality or equivalent and water was of resistivity >18 M Ω cm (Elga PureLab Maxima).

Preparation of ferrocene-terminated oligonucleotide

Synthesis of ferrocene-terminated oligonucleotide, **Fc-E**, from the amine-terminated oligonucleotide, **E**, and ferrocene carboxylic acid *N*-succinimide ester (Molecular Sensing) was based on the method of Ihara and coworkers.¹⁶ Crude purification of oligonucleotide product was by gel-filtration with a Pharmacia NAP-10 column (0.1 M triethylammonium acetate, pH 6.8). The void volume was subject to reverse-phase HPLC with a Luna 5 μ C18(2) column (150 \times 4.6 mm) employing mobile phases of 0.1 M triethylammonium acetate, pH 6.8 and 10% acetonitrile (10 to 30% acetonitrile applied over 20 min at 1 mL min⁻¹). Elution of **Fc-E** at 13 min was detected by an increase of absorbance at 260 nm. Purified **Fc-E**

† Electronic supplementary information (ESI) available: HPLC characterisation of ferrocene-labelled oligonucleotides. See <http://www.rsc.org/suppdata/an/b4/b413556c/>

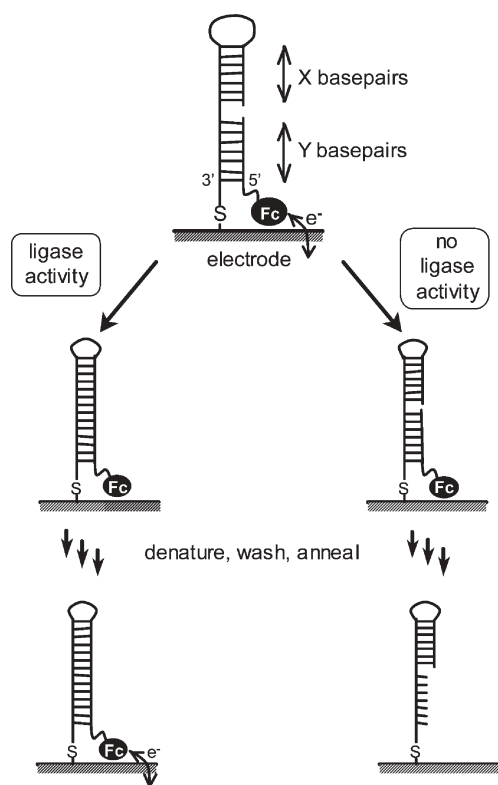


Fig. 1 Concept for the utilisation of tethered, nicked DNA hairpins for the voltammetric detection of DNA ligase activity. Nicked hairpins are identified throughout the text with an 'X + Y' nomenclature that relates the position of the nick to the number of basepairs in the stem of the hairpin as indicated in the upper panel.

was concentrated and exchanged into 20 mM HEPES, 1 M NaCl, pH 7.0. **Fc-E** concentration was estimated using $\epsilon_{260\text{nm}} = 135\,700\text{ M}^{-1}\text{ cm}^{-1}$ calculated from the values of 15 400, 11 500, 8 700 and 7 400 $\text{M}^{-1}\text{ cm}^{-1}$ for the A, G, T and C respectively and 9 500 $\text{M}^{-1}\text{ cm}^{-1}$ for the ferrocene label. Control experiments performed with **D** showed no evidence of a reaction with ferrocene carboxylic acid *N*-succinimide ester

and confirmed the amine terminus as the site of ferrocene addition to **E**.

Preparation of nicked DNA hairpins

Nicked hairpins carrying an identical six base loop but with variable stem lengths and positioning of the nick within the stem were prepared by hybridisation of pairs of partially complementary oligonucleotides. The resultant hairpins were distinguished by the nomenclature 'X+Y' where the nick is positioned X basepairs from the loop and Y basepairs from the foot of the hairpin, Fig. 1. Thus, hybridisation of oligonucleotides **A** and **B** (Table 1) forms '8+6', **A** and **C** forms '14+6', **E** (or **F**) and **G** forms '8+12', **E** (or **F**) and **H** (or **J**) forms '14+12'. Oligonucleotides were dissolved in 90 mM Tris-borate, 10 mM EDTA, pH 8.3 (TBE) buffer. Those (**B**, **C**, **G**, **H** and **J**) defining the 5' side of the nick were phosphorylated by T4 polynucleotide kinase (AbGene), purified by ethanol precipitation and resuspended in TBE buffer. Nicked DNA hairpins were formed with equimolar concentrations (typically 19 μM) of the appropriate oligonucleotides, heated at 90–100 °C for 5 min and slow-cooled to room temperature.

Characterisation of DNA hairpins in solution

Nicked DNA hairpins (50 pmoles) were incubated with DNA ligase (20 pmoles) in 10 μL of 26 μM NAD^+ , 10 mM MgCl_2 , 25 $\mu\text{g mL}^{-1}$ bovine serum albumin, 10 mM dithiothreitol, 50 mM Tris-HCl, pH 8 at 25 °C for 1 h. Control experiments were performed under identical conditions without the addition of DNA ligase. For product analysis samples were subjected to non-denaturing electrophoresis (15% polyacrylamide gel, 80 V, 6 h, TBE buffer) or combined with an equal volume of formamide loading buffer, heated to 95 °C and subjected to denaturing electrophoresis (15% polyacrylamide-urea gel, 300 V, 1 h, TBE buffer). Reaction products were visualised and quantitated using a Molecular Dynamics Storm phosphorimager.

Table 1 Summary of oligonucleotides used in this work^a

Oligonucleotide	Sequence
A	5' F1 -TGACTC 3'
B	3' ACTGAGCGAGT <u>GCGAGTGT</u> ACGCACTCG 5'
C	3' ACTGAGCGATGGACAGT <u>GCGAGTGT</u> ACGCACTGTCCATCG 5'
D	5' TGAACCTAGCTC 3'
E	5' H₂N -TGAACCTAGCTC 3'
F	5' F1 -TGAACCTAGCTC 3'
G	3' ACTTGAATCGAGCGAGT <u>GCGAGTGT</u> ACGCACTCG 5'
H	3' ACTTGAATCGAGCGATGGACAGT <u>GCGAGTGT</u> ACGCACTGTCCATCG 5'
J	3' RSS -ACTTGAATCGAGCGATGGACAGT <u>GCGAGTGT</u> ACGCACTGTCCATCG 5'

^a F1 = fluorescein label, RSS = disulfide label with $(\text{CH}_2)_6$ linker to oligonucleotide, H_2N = amino label with $(\text{CH}_2)_6$ linker to oligonucleotide. For oligonucleotides that can form a hairpin structure the bases of the loop are underlined.

Preparation and voltammetric characterisation of tethered DNA hairpins

Gold electrodes of *ca.* 4 mm diameter were prepared on glass microscope slides by vacuum evaporation of ~ 20 nm chromium followed by 180 nm gold. Immediately prior to use electrodes were cleaned with warm (60–70 °C) piranha solution (70% concentrated sulfuric acid, 30% peroxide solution (30%)) for 30 min, caution: piranha solution may react violently with organics, rinsed thoroughly with water and dried with a flow of N_2 gas. Typically 1 μ L of 20 μ M hairpin, 20 mM HEPES, 1 M NaCl, pH 7.0 was placed on an electrode and left in a humidified chamber at room temperature for 3 to 16 h as desired. The electrode was rinsed thoroughly with water then 20 mM HEPES, 1 M NaCl, pH 7.0. Non-specific interactions between the thiolated DNA and the gold surface were removed by exposure to 1 M mercaptoethanol for 2 h.¹⁷ Finally, electrodes were rinsed with 1 M $NaClO_4$, 25 mM Na_2HPO_4/NaH_2PO_4 , pH 7.0 and stored in this buffer until use, typically within 2 days of preparation. Ligations were performed with 2.6 μ M DNA ligase in 37 μ M NAD^+ , 5.7 mM $MgCl_2$, 0.15 mM mercaptoethanol, 7 mM HEPES, pH 7.5 for 90 or 160 min at 37 °C. Denaturation of the immobilised hairpins with 0.5% sodium dodecyl sulfate, 0.5 M NaOH was at room temperature.

Electrochemical measurements were performed with a three-electrode cell configuration housed in a N_2 -filled chamber (atmospheric $O_2 < 2$ ppm). A KCl saturated Ag/AgCl reference electrode contacted the cell through a Luggin tip and a platinum wire formed the counter electrode. Voltammetry was performed at 23 °C with an Autolab 30 potentiostat under the control of GPES software. Potentials are reported relative to SHE by addition of 197 mV to that measured.

Results

Selection of a nicked DNA hairpin

Successful implementation of our concept for the ligase assay is dependent on a number of factors. Good signal intensity requires that nicked hairpins are the predominant species on the electrode, *i.e.* the dissociation constant describing separation of its two strands must be low. This can be readily achieved with 'long' oligonucleotides but should be balanced by a desire to keep the hairpin short for economic reasons. The hairpin must also display the nick in such a way that it is accessible to the ligase. The dimensions of DNA ligases suggest that the electrode surface and hairpin loop should be separated by at least 60 Å for successful ligation in the hairpin stem.¹⁸ To maximise the chance that the immobilised hairpins would stand proud of the surface a mercaptohexyl linker would be positioned at the 3' terminus.¹⁹

With these points in mind the properties of four nicked hairpins were screened using the standard assay for activity of the NAD^+ -dependent ligase from *E. coli* (LigA).^{14,15} Product characterisation in this solution phase assay is by gel electrophoresis so the nicked hairpins carried a 5' fluorescein label for visualisation. A stem of fourteen base pairs (*ca.* 48 Å) combined with a fully extended mercaptohexyl linker (*ca.* 11 Å)

is likely to be the minimum length that could support ligation. Therefore, hairpins with stems of 14, 20 and 26 base pairs were designed in which the position of the nick relative to the loop was varied. These hairpins are referred to by an 'X+Y' nomenclature where X represents the number of base pairs between the loop and the nick, and Y represents the number of base pairs between the nick and the foot of the stem, Fig. 1.

The extent of ligation achieved in each hairpin was assessed by gel electrophoresis under denaturing conditions, Fig. 2A. Successful ligation is indicated by greater retardation of the longer fluorescein labelled DNA strands that are present in the samples. Quantitation of the extent of ligation showed that the '8+12' and '14+12' hairpins were more effectively ligated, 49 and 41% respectively, than those hairpins with six basepairs between the nick and the foot of the hairpin, *i.e.*, '8+6' (6%) and '14+6' (35%). That ligation is more effective with 12 bases, *i.e.*, 41 Å, between the nick and the loop shows that the length of DNA required for productive association of the ligase and DNA is greater than that predicted from the crystallographic dimensions of the enzyme.

Further analysis of the suitability of the nicked hairpins for the desired assay through assessment of their structural

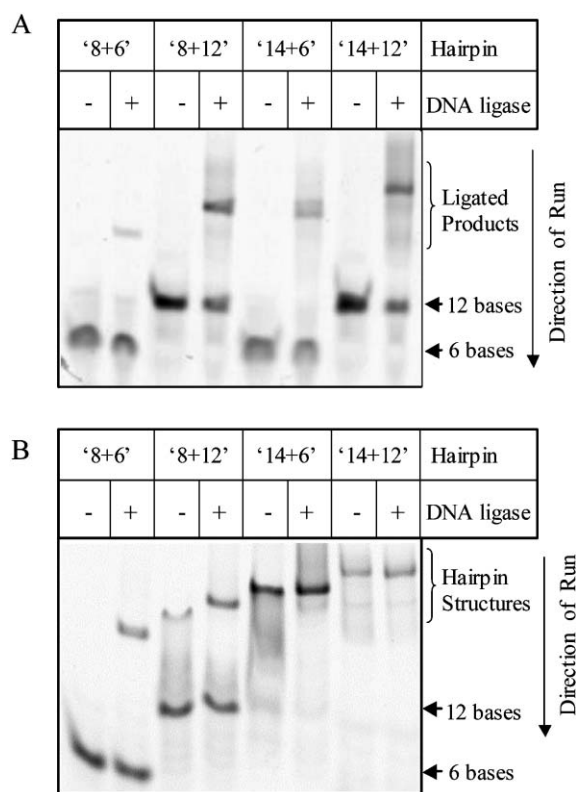


Fig. 2 Ligation of nicked DNA hairpins by the NAD^+ -dependent DNA ligase from *E. coli*. *In vitro* incubations using the indicated DNA substrate were performed without or with DNA ligase (lanes shown as “-” and “+”, respectively). (A) To detect ligation of the DNA ends, samples were electrophoresed on a denaturing 15% polyacrylamide gel. (B) To analyse the structural integrity of the DNA hairpins during the reaction, samples were electrophoresed on a 15% non-denaturing polyacrylamide gel. For both gels, arrows indicate the 6- and 12-base oligonucleotides containing the 5'-fluorescein from the unligated, nicked hairpins.

integrity was provided by gel electrophoresis under non-denaturing conditions, Fig. 2B. A high affinity between the oligonucleotides forming the nicked hairpins will produce a high proportion of hybridised oligonucleotides, which will have different electrophoretic mobility compared to non-hybridised DNA. Furthermore, the mobility of this single band should be unaffected by ligation. This is seen for the '14+12' sample. By contrast the '8+12' and '8+6' samples show a much greater population of fluorescein labelled single-strand, especially for substrates not exposed to DNA ligase. The presence of some single stranded fluorescein-terminated oligonucleotide is suggested by smearing in the '14+6' sample prior to ligase exposure.

Due to the propensity of the '14+12' sample to form nicked hairpins that are amenable to ligation this substrate was selected for exploration of the feasibility of ligating tethered nicked hairpins. The 3' mercaptohexyl linker was omitted from hairpins used in the initial screening of nicked hairpin behaviour. Control experiments established that '14+12' hairpins differing only in the presence or absence of the 3' linker showed indistinguishable ligation efficiencies in assays comparable to those described above (data not shown).

Ligation of tethered, nicked DNA hairpins

Gold electrodes coated with 3' mercaptohexyl terminated '14+12' bearing 5' ferrocene gave cyclic voltammograms containing a pair of peaks, Fig. 3. These peaks could be attributed to the oxidation and reduction of ferrocene since they were absent when experiments were performed with oligonucleotides lacking a ferrocene label. The peak areas are similar for the oxidative and reductive processes indicating reversibility of the electrode reaction. Integration of the peaks and the assumption of a flat electrode yields coverages on the order of 1×10^{14} molecules cm^{-2} . Given that the maximum theoretical density of hydrated duplex DNA is *ca.* 3×10^{13} molecules cm^{-2} the observed value is most likely to reflect roughness of the electrode surface (see for example ref. 20). Under these conditions interactions between neighboring molecules may account for deviation of the peak shapes from those predicted for identical, independent redox systems and that are observed from less densely packed assemblies of similar molecules.¹³ Indeed the relative distortion of the wavepair in Fig. 3B as compared to Fig. 3A may reflect the higher electroactive coverage of the electrode in the former experiment.

Exposure of hairpin coated electrodes to conditions that denature the duplex stem followed by rinsing to remove non-covalently bound material resulted in loss of >98% of the ferrocene response, Fig. 3A. Signals were lost after 5 min exposure to the denaturation conditions and longer incubations produced no further change to the voltammetric response. In contrast, a clear ferrocene signal was retained by electrodes exposed to ligase prior to exposure to denaturing conditions, Fig. 3B. These peaks were retained essentially unaltered after a further 10 and 30 min exposure to denaturation conditions. Thus, DNA ligase was able to perform covalent attachment of ferrocene to the electrode through ligation of the tethered nicked hairpin substrate.

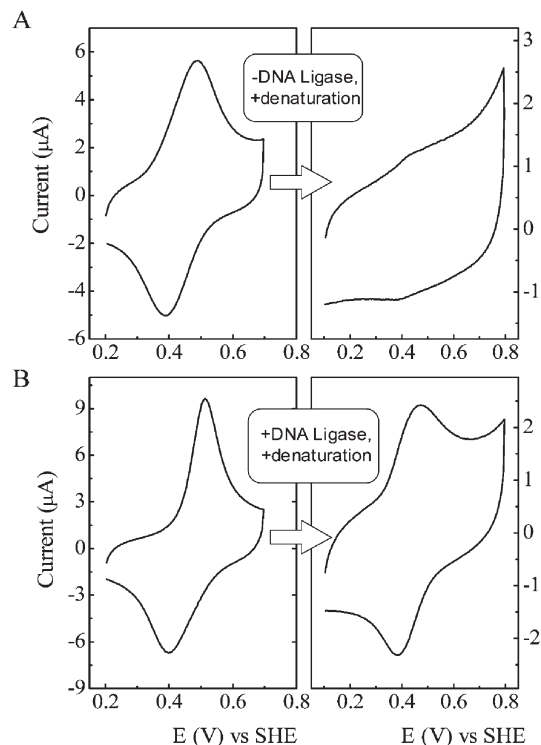


Fig. 3 The influence of *E. coli* NAD⁺-dependent DNA ligase on cyclic voltammetry from gold electrodes coated with ferrocene terminated nicked hairpins. (A) Voltammograms recorded before and after exposure to denaturation buffer and washing to remove non-covalently bound material. (B) Voltammograms recorded before and after exposure to DNA ligase, denaturation buffer and washing as described for (A). Cyclic voltammetry measured in 0.1 M NaClO₄ at a scan rate of 0.1 V s⁻¹, other details as described in the Experimental protocols section.

The voltammetric peak areas from several experiments with hairpin-coated electrodes are presented in Fig. 4. The approximately 10-fold variation of the initial coverage of the electrodes was achieved through variation of the time allowed for hairpin adsorption. None of the electrodes exposed to DNA ligase retained 100% of their initial signal intensity. This is in line with the results from electrophoretic analysis, Fig. 2A (and see also ref. 14). However, the number of molecules retained after ligation was remarkably similar in all experiments at *ca.* 5×10^{13} molecules cm^{-2} (leading to an apparent ligation efficiency between 50 and 20% across the electrodes studied as illustrated in Fig. 4) This suggests that a constant population of tethered molecules is accessible to ligase perhaps due to roughness of the surface that renders some nicks inaccessible to the active site of the enzyme. An alternative explanation that the dense substrate packing may prevent access to certain populations of the tethered substrate seems less likely as this would result in lower populations of ligated product at electrodes with higher surface coverage, which was not observed.

Discussion

We have provided proof of concept for a novel electrochemical assay of DNA ligase activity. The enzyme used in our studies, *E. coli* LigA, has served as the paradigm for elucidation of the

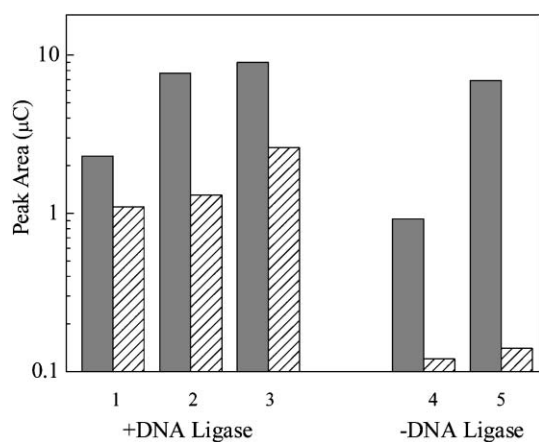


Fig. 4 Quantification of the influence of *E. coli* DNA ligase on cyclic voltammetric peak areas displayed by ferrocene terminated hairpins tethered to gold electrodes. Solid bars: initial peak areas displayed by five, independently prepared electrodes coated with ferrocene terminated nicked hairpins. Striped bars: peak areas after exposure to ligase reaction buffer that included or omitted DNA ligase, exposure to denaturation buffer and washing to remove non-covalently bound material.

properties of NAD⁺-dependent DNA ligases, a family of proteins of molecular weight *ca.* 74 kDa and extensive amino acid sequence homology.⁶ The smaller ATP-dependent DNA ligases (MW *ca.* 31 kDa) also form a homologous group and we have found that the enzyme from bacteriophage T4 also readily ligates the '14+12' hairpin.²¹ Thus, this hairpin provides a suitable substrate for assaying DNA ligases from many different organisms. This generality of substrate for DNA ligases of different types is in large part due to the relative insensitivity of DNA ligase action to the sequence context of the nick.²² This property contrasts to that of many other DNA processing enzymes. However, DNA hairpins designed to contain appropriate DNA sequences should allow analysis of a number of DNA processing enzymes through an approach similar to that described here. For example, the activity of nucleases that introduce nicks into duplex DNA should be detected by reversing the assay concept we describe.

In terms of DNA ligase analysis the electrochemical methodology described here produces results in good qualitative agreement with those from the traditional electrophoretic analysis. Importantly, the simplicity and success of the voltammetric end-point assay demonstrates that DNA ligase action can be studied in proximity to a gold surface. This provides a framework from which necessarily more complex methods can be confidently pursued for resolution of enzyme action *in real-time*. When conditions for 100% ligation of the tethered hairpins have been defined, methods such as *in situ* scanning probe microscopies, surface plasmon resonance and attenuated total internal reflection-Fourier transform infrared spectroscopies should provide such information with resolution at the molecular and sub-molecular levels (for example see refs. 23–25). It may also be possible to exploit the time-base of the electrochemical analysis to monitor specifically changes in the conformation and/or rigidity of the DNA duplex during ligation. This could be done by positioning the redox reporter on the side of the nick remote from the electrode to allow the rates of electron conduction through the π -stack of the DNA

duplex to be measured.²⁶ The success of such experiments will afford information that is difficult to access by current assays of ligation and experiments pursuing these various possibilities are now underway in our laboratories.

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