BIOELECTRONIC CIRCUITS, SYSTEMS AND METHODS FOR PREPARING AND USING THEM

FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

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BACKGROUND

The use of streptavidin-biotin linkages for building molecular assemblies is well-known in the art with many reagents commercially available. It is also well-know that chemical coupling of proteins to electrodes enhances electron transfer between a metal electrode and a protein. This can take the form of a specific covalent linkage[1] or, alternately, functionalization of the electrode with a small molecule (such as an amino acid) that renders the electrode hydrophilic and thus capable of forming hydrogen bonds with a protein.[2]

While it is known that some proteins are electrically conductive over extraordinarily long distances[2-4] it has recently become clear that this conductivity is only apparent when specific chemical contacts are made between electrodes and proteins, and, in particular, connections based on the interactions of cognate ligands that interact with the hydrophobic interior of the protein that has evolved to interact with them.[5] Furthermore, this recent work showed that high electronic conductivity (nS over distances of many - 2 to 20 - nm) appears to be a common property of proteins, not just proteins that have evolved to carry out electron transfer functions. Such behavior is referred to herein as quasi-metallic conduction. Finally, this work also showed that the binding of a specific ligand molecule (itself bound to an electrode by a thiol linkage) to a protein, produced a better contact to the protein than direct attachment of the protein itself to the electrode via a surface thiol modification of the protein. Thus, ligands that may bind via weak and reversible bonds, but that, in doing so contact the hydrophobic interior of the protein, are better electrical connectors than strong, irreversible covalent bonds to residues on the hydrophilic exterior of the protein. The most robust recipe for making an electrical contact between a protein and an electrode, or between one protein and another, is to use the specific chemical contact that the protein has evolved to make: that is the ligand-receptor interactions. Furthermore, there are theoretical grounds[6] for believing that the interior of proteins manifests quasi metallic properties as an electronic transport material. This is consistent with Zhang et al.'s findings of conductance that is too high to measure (i.e., contact limited) in a series of proteins that are not electron transfer proteins.[5] The same properties exist in electron transfer

proteins (where they might be expected on the basis of biological need). Interestingly, all the functional proteins examined have this quasi-metallic property, even though there is not, at present, known evolutionary role for it in the proteins that do not carry out electron transport functions.

SUMMARY

The present disclosure provides bioelectronic circuits, systems and methods for preparing and using them.

In one embodiment, bioelectronic circuits are provided. In one embodiment of this embodiment, the bioelectronic circuit comprises (a) at least one electrode, (b) at least one ligand that is specific for a protein, wherein the ligand is modified so that it attaches to the at least one electrode, and (c) at least one protein that binds the ligand, thereby forming an electronic contact between the electrode and the protein.

Disclosed herein is a bioelectronic circuit comprising: (a) a first electrode and a second electrode, (b) a first ligand and a second ligand that are specific for a protein, wherein the first and second ligands are modified so that the first ligand attaches to the first electrode and the second ligand attaches to the second electrode, and (c) a protein that is modified to bind the first and second ligands, wherein the binding of the protein to the first and second ligands forms an electronic contact between the electrode and the protein, wherein the protein comprises at least one protein selected from the group consisting of i) IgE Anti-DNP, IgG Anti-HIV, IgG Anti-Ebola, Fab Anti-Ebola, αVβ3 Integrin, and streptavidin, or ii) a polymerase, endonuclease, helicase and a proteosome protein.In one embodiment, the bioelectronic circuit comprises (a) a first and a second electrode, (b) a first and a second ligand that are specific for a protein, wherein the ligands are modified so that the first ligand attaches to the first electrode and the second ligand attaches to the second electrode, and (c) a protein that is modified to bind the first and the second ligands, wherein the binding of the protein to the first and the second ligands forms an electronic contact between the electrode and the protein.

In one embodiment, there is a bioelectronic circuit comprising: (a) a first electrode and a second electrode, (b) a first ligand and a second ligand that are specific for a protein in a protein assembly, wherein the first and second ligands are modified so that the first ligand attaches to the first electrode and the second ligand attaches to the second electrode, and (c) a protein assembly comprising a defined number of proteins that is modified to bind the first and the second ligands, wherein the binding of a protein in the protein assembly to the first and the second ligands forms an electronic contact between the electrode and the protein assembly.

In one embodiment, the bioelectronic circuit comprises (a) a first and a second electrode, (b) a first ligand that is specific for a protein, wherein the first ligand is modified so that it attaches to the first electrode, (c) a protein that binds the first ligand and is modified to bind a second ligand, and (d) a second ligand that binds the protein and is modified so that it attaches to the second electrode, thereby forming an electronic contact between the first and second electrodes and the protein.

Disclosed herein is a bioelectronic circuit comprising: (a) a first electrode and a second electrode, (b) a first ligand that is specific for a protein, wherein the first ligand is modified so that it attaches to the first electrode, (c) a protein that binds the first ligand and is modified to bind a second ligand, and (d) a second ligand that binds the protein and is modified so that it attaches to the second electrode, thereby forming an electronic contact between the first and second electrodes and the protein wherein the protein comprises at least one protein selected from the group consisting of i) IgE Anti-DNP, IgG Anti-HIV, IgG Anti-Ebola, Fab Anti-Ebola, αVβ3 Integrin, and streptavidin, or ii) a polymerase, endonuclease, helicase and a proteosome protein.

In one embodiment, there is a bioelectronic circuit comprises (a) a first electrode and a second electrode, (b) a first protein, wherein the first protein is attached to the first electrode via biotin-streptavidin interactions, (c) a second protein, wherein the second protein is attached to the first protein via biotin-streptavidin interactions and is modified to bind a ligand, (d) a ligand that binds the second protein and is modified so that it attaches to the second electrode, thereby forming an electronic contact between the first and second electrodes and the first and second protein.

In one embodiment, there is a bioelectronic circuit comprises (a) a first electrode and a second electrode, (b) a first protein and a second protein, wherein one of, or both, the first and the second proteins are attached to the first and second electrodes through biotin-streptavidin interactions, thereby forming an electronic contact between the first and the second electrodes and one of, or both, the first and the second proteins.

In one embodiment, there is a bioelectronic circuit comprises (a) a first electrode and a second electrode, (b) a first protein, wherein the first protein is attached to the first electrode via biotin-streptavidin interactions, (c) a second protein, wherein the second protein is attached to the first protein via biotin-streptavidin interactions and is attached to the second electrode via biotin-streptavidin interactions, (d) a third protein, wherein the third protein is attached to the first and the second proteins via biotin-streptavidin interactions, thereby forming an electronic contact between the first and second electrodes and the first, second and third proteins.

In one embodiment, there is a bioelectronic circuit comprises (a) a first electrode, (b) a first protein, wherein the first protein is attached to the first electrode via biotin-streptavidin interactions, (c) a second protein, wherein the second protein is attached to the first protein via biotin-streptavidin interactions and is attached to the second electrode via biotin-streptavidin interactions, (d) a second electrode in contact with an electrolyte and connected to the first electrode, (e) a means for applying a voltage bias, (f) a means for sensing current, thereby forming an electronic contact between the first and second electrodes and the first and second proteins.

In one embodiment, the bioelectronic circuit is as herein described wherein the protein comprises two or more Avitag sequences placed at the surface of the protein and no more than 10 amino acid residues from tyrosines, tryptophans or histidines within the protein.

Disclosed herein is a bioelectronic circuit comprises (a) a first electrode and a second electrode, (b) a first ligand and a second ligand that are specific for a protein, wherein the first and second ligands are modified so that the first ligand attaches to the first electrode and the second ligand attaches to the second electrode, (c) a first protein and a second protein that bind the first ligand and the second ligand and (d) a third protein that is modified to bind the first and the second proteins, wherein the binding of the third protein to the first and the second proteins forms an electronic contact between the electrode and the protein.

Disclosed herein is a bioelectronic circuit comprises (a) a first electrode and a second electrode, (b) a first ligand and a second ligand that are specific for a protein, wherein the first and second ligands are modified so that the first ligand attaches to the first electrode and the second ligand attaches to the second electrode, (c) at least a first protein and a second protein that bind the first ligand and the second ligand, and may be coupled to further proteins via ligands, extending the range over which conduction is obtained.

In another embodiment, systems for electrical measurement of protein activity are provided. The system comprises (a) a bioelectronic circuit as herein described, (b) a means for applying a bias between the first and second electrode, and (c) a means for detecting the current through the bioelectronic circuit.

Disclosed herein is a system for electrical measurement of protein activity comprising: (a) a bioelectronic circuit as described herein, (b) a means for applying a bias between the first and second electrode, and (c) a means for detecting the current through the bioelectronic circuit.

In another embodiment, methods of preparing bioelectronic circuits are provided. In one embodiment, the method comprises (a) attaching at least one streptavidin molecule to at least one electrode, (b) introducing a biotinylated protein to the streptavidin-electrode from step (a) to form a complex comprising the at least one electrode, the at least one streptavidin molecule and the biotinylated protein.

In one embodiment, there is a method of preparing a bioelectronic circuit, the method comprising: (a) attaching at least one streptavidin molecule to at least one electrode, (b) introducing a biotinylated protein to the streptavidin-electrode from step (a) to form a complex comprising the at least one electrode, the at least one streptavidin molecule and the biotinylated protein.

Disclosed herein is a method for detecting the activity of a polymerase, the method comprising introducing a solution of DNA template and nucleotriphosphates to any of the bioelectronic circuits as herein described, wherein polymerized product indicates that the polymerase is active.

In another embodiment, methods for detecting the activity of protein. The method comprises introducing a substrate of the protein to any of the bioelectronic circuit herein described and detecting electrical changes.

Disclosed herein is a method for detecting the activity of protein, the method comprising introducing a substrate of the protein to the bioelectronic circuit as described herein and detecting electrical changes.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 shows the chemical structure of the molecule bis-biotin cystamine.

FIG. 2 shows the chemical structure of the reduced version of the molecule shown in **FIG. 1**.

FIG. 3 shows a schematic diagram of a bioelectronic circuit using streptavidin wires to wire a biotin-modified polymerase into the circuit.

FIG. 4 shows a gel assay demonstrating that biotinylated polymerase bound to two streptavidin molecules is active and capable of efficient enzyme action.

FIG. 5A and **FIG. 5B** show a comparison of the measured conductance distributions for mono-biotinylated and bis-biotinylated polymerases.

FIG. 6A and **FIG. 6B** show a recording of current vs time for a polymerase molecule wired into a bioelectronic circuit with streptavidin.

FIG. 7A and **FIG. 7B** show a comparison of measured conductance distributions for thiolated streptavidin bound with biotin and directly coupled to electrodes with that for wild-type streptavidin bound to electrodes with the thiolated biotin shown in **FIG. 2**. Here, the biotin is shown exaggerated in size for clarity.

FIG. 8 shows a schematic diagram of orienting circuit elements using more than one selective ligand to form a biomolecular AND gate.

FIG. 9A and **FIG. 9B** show cloning constraints on the choice of protein sequence modification.

FIG. 10A and **FIB. 10B** shows examples of protein sequences for phi29 polymerase modified to contain two Avitag linkers showing how the incorporation of charged residues can cause misfolding.

FIG. 11A and **FIG. 11B** show conductance distributions measured for two polymerases with and without a flexible linker sequence as wired into a circuit with biotin-streptavidin linkers.

FIG. 12 shows a schematic diagram of a biomolecular OR gate.

FIG. 13 shows a schematic diagram of a biomolecular three-state gate.

FIG. 14 shows a schematic diagram of a single electrode circuit in which the second contact is formed by a redox-active protein immersed in an electrolyte solution.

FIG. 15 shows a bioelectronic circuit in which an unmodified streptavidin is tethered to an electrode by the thio-biotin of **FIG. 2**

FIG. 16 shows a bioelectronic circuit in which the coupling is extended in distance by incorporating a second streptavidin and the bis-biotin molecule of **FIG. 1**.

DETAILED DESCRIPTION OF THE INVENTION

The invention includes the following:

1. A bioelectronic circuit as shown and described.

2. A system for electrical measurement of protein activity as shown and described.

3. A method for detecting the activity of a protein as shown and described.

4. A bioelectronic circuit comprising:

(a) at least one electrode,

(b) at least one ligand that is specific for a protein, wherein the ligand is modified so that it attaches to the at least one electrode, and

(c) at least one protein that binds the ligand, thereby forming an electronic contact between the electrode and the protein.

5. The bioelectronic circuit according to the above 4, wherein the electrode comprises a metal selected from the group consisting of palladium, platinum or gold.

6. The bioelectronic circuit according to the above 4 or 5, wherein the ligand is selected from the group consisting of $HSCH_2CH_2$-dinitrophenol, CALDRWEKIRLR (SEQ ID NO: 1), CHNTPVYKLDISEATQV (SEQ ID NO: 2), cyclic RGDfC, thiolated-streptavidin, and $HSCH_2CH_2$-biotin.

7. The bioelectronic circuit according to any of the above 4 to 6, wherein the protein is selected from the group consisting of IgE Anti-DNP, IgG Anti-HIV, IgG Anti-Ebola, Fab Anti-Ebola, $\alpha_V\beta_3$ Integrin, and streptavidin.

8. The bioelectronic circuit according to any of the above 4 to 6, further comprising wherein the protein is a polymerase, endonuclease, helicase or a proteosome.

9. The bioelectronic circuit according to the above 4, wherein the at least one ligand is modified to comprise a thiol, amine, disulfide or cyanide moeity.

10. A bioelectronic circuit comprising:

(a) a first and a second electrode,

(b) a first and a second ligand that are specific for a protein, wherein the ligands are modified so that the first ligand attaches to the first electrode and the second ligand attaches to the second electrode, and

(c) a protein that is modified to bind the first and the second ligands, wherein the binding of the protein to the first and the second ligands forms an electronic contact between the electrode and the protein.

11. The bioelectronic circuit of the above 10, wherein the ligand is a thiolated streptavidin.

12. The bioelectronic circuit of the above 10 or 11, wherein the protein is modified to contain biotin.

13. The bioelectronic circuit of any of the above 10 to 12, wherein the protein is bis-biotinylated polymerase.

14. A bioelectronic circuit comprising:

(a) a first and a second electrode,

(b) a first ligand that is specific for a protein, wherein the first ligand is modified so that it attaches to the first electrode,

(c) a protein that binds the first ligand and is modified to bind a second ligand, and

(d) a second ligand that binds the protein and is modified so that it attaches to the second electrode, thereby forming an electronic contact between the first and second electrodes and the protein.

15. The bioelectronic circuit of the above 14, wherein the binding site on the protein to the first ligand is in proximity to a flexible linker sequence.

16. The bioelectronic circuit of the above 14, wherein the second binding site on the protein to the second ligand is in proximity to a flexible linker sequence.

17. The bioelectronic circuit of the above 15 or 16, wherein the flexible linker sequence comprises GNSTNGTSNGSS (SEQ ID NO: 3).

18. A bioelectronic circuit comprising:

(a) a first and a second electrode,

(b) a first protein, wherein the first protein is attached to the first electrode via biotin-streptavidin interactions,

(c) a second protein, wherein the second protein is attached to the first protein via biotin-streptavidin interactions and is modified to bind a ligand,

(d) a ligand that binds the second protein and is modified so that it attaches to the second electrode, thereby forming an electronic contact between the first and second electrodes and the first and second protein.

19. The bioelectronic circuit according to the above 18, wherein the first protein comprises two biotins.

20. The bioelectronic circuit of the above 19, wherein the second protein comprises two biotinylated Avitag sequences.

21. A bioelectronic circuit comprising:

(a) a first and a second electrode,

(b) a first and a second protein, wherein one of, or both, the first and the second proteins are attached to the first and second electrodes through biotin-streptavidin interactions, thereby forming an electronic contact between the first and the second electrodes and one of, or both, the first and the second proteins.

22. The bioelectronic circuit of the above 21, wherein the first binding site comprises one or more residues that can be biotinylated.

23. A bioelectronic circuit comprising:

(a) a first and a second electrode,

(b) a first protein, wherein the first protein is attached to the first electrode via biotin-streptavidin interactions,

(c) a second protein, wherein the second protein is attached to the first protein via biotin-streptavidin interactions and is attached to the second electrode via biotin-streptavidin interactions,

(d) a third protein, wherein the third protein is attached to the first and the second proteins via biotin-streptavidin interactions, thereby forming an electronic contact between the first and second electrodes and the first, second and third proteins.

24. A bioelectronic circuit comprising:

(a) a first electrode,

(b) a first protein, wherein the first protein is attached to the first electrode via biotin-streptavidin interactions,

(c) a second protein, wherein the second protein is attached to the first protein via biotin-streptavidin interactions and is attached to the second electrode via biotin-streptavidin interactions,

(d) a second electrode in contact with an electrolyte and connected to the first electrode,

(e) a means for applying a voltage bias,

(f) a means for sensing current, thereby forming an electronic contact between the first and second electrodes and the first and second proteins.

25. A method of preparing a bioelectronic circuit, the method comprising:

(a) attaching at least one streptavidin molecule to at least one electrode,

(b) introducing a biotinylated protein to the streptavidin-electrode from step (a) to form a complex comprising the at least one electrode, the at least one streptavidin molecule and the biotinylated protein.

26. A method for detecting the activity of a polymerase, the method comprising introducing a solution of DNA template and nucleotriphosphates to the bioelectronic circuit of the above 4, wherein polymerized product indicates that the polymerase is active.

27. A method for detecting the activity of protein, the method comprising introducing a substrate of the protein to the bioelectronic circuit of the above 4 and detecting electrical changes.

28. A system for electrical measurement of protein activity comprising:

(a) a bioelectronic circuit as herein described,

(b) a means for applying a bias between the first and second electrode, and

(c) a means for detecting the current through the bioelectronic circuit.

29. A bioelectronic circuit as herein described wherein the protein comprises two or more Avitag sequences placed at the surface of the protein and no more than 10 amino acid residues from tyrosines, tryptophans or histidines within the protein.

30. A bioelectronic circuit comprising:

(a) a first and a second electrode,

(b) a first and a second ligand that are specific for a protein, wherein the ligands are modified so that the first ligand attaches to the first electrode and the second ligand attaches to the second electrode,

(c) a first protein and a second protein that bind the first ligand and the second ligand, and

(d) a third protein that is modified to bind the first and the second proteins, wherein the binding of the third protein to the first and the second proteins forms an electronic contact between the electrode and the protein.

31. The bioelectronic circuit of the above 30, wherein the first and second proteins are streptavidin

32. The bioelectronic circuit of the above 30, wherein the first and second ligands are biotin.

33. The bioelectronic circuit of the above 30, wherein the third protein is biotinylated.

34. A bioelectronic circuit comprising:

(a) a first and a second electrode,

(b) a first and a second ligand that are specific for a protein, wherein the ligands are modified so that the first ligand attaches to the first electrode and the second ligand attaches to the second electrode, and

(c) at least a first protein and a second protein that bind the first ligand and the second ligand, and may be coupled to further proteins via ligands, extending the range over which conduction is obtained.

DEFINITIONS

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as those commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or

testing of the present disclosure, suitable methods and materials are described below. The materials, methods and examples are illustrative only, and are not intended to be limiting. All publications, patents and other documents mentioned herein are incorporated by reference in their entirety.

Throughout this specification, the word "comprise" or variations such as "comprises" or "comprising" will be understood to imply the inclusion of a stated integer or groups of integers but not the exclusion of any other integer or group of integers.

The term "a" or "an" may mean more than one of an item.

The terms "and" and "or" may refer to either the conjunctive or disjunctive and mean "and/or".

The term "about" means within plus or minus 10% of a stated value. For example, "about 100" would refer to any number between 90 and 110.

BIOELECTRONIC CIRCUITS

The present disclosure provides a bioelectronic circuit. The bioelectronic circuit comprises (a) at least one electrode, (b) at least one ligand that is specific for a protein, wherein said ligand is modified so that it attaches to the at least one electrode, and (c) at least one protein that binds the at least one ligand, thereby forming an electronic contact between the electrode and the protein.

Depending on the application, the bioelectronic circuit comprises one or two electrodes in direct contact with proteins. In the case where only one electrode is in contact with the proteins, the circuit is completed by means of ion currents that flow between a redox-active protein and a remote electrode. In one embodiment, the bioelectronic circuit comprises one electrode. In another embodiment, bioelectronic circuit comprises two electrodes. In some aspects of this embodiment, the second electrode is not separated from the first electrode by an electrolyte.

The at least one electrode comprises a noble metal. In one embodiment, the at least one electrode comprises a noble metal selected from the group consisting of palladium, gold, and platinum. In another embodiment, the at least one electrode is palladium. In another embodiment, the at least one electrode is gold. In another embodiment, the at least one electrode is platinum. In addition to the at least one electrode, the bioelectronic circuit comprises a ligand that is specific for a protein and is modified so that it attaches to the at least one electrode.

The ligand can be modified to contain a thiol termination at one end for coupling to metals, as described by Zhang et al.[5] Examples of ligands are peptide epitopes for antibodies comprising a cysteine residue at one end, recognition peptides (such as the RGD peptide for binding integrin comprising a cysteine) and small molecules to which proteins have been selected to bind (such as an IgE molecule that binds dintitrophenyl and comprising a thiol).

Exemplary ligands that are specific for a protein and modified to attach to an electrode include, but are not limited to, **HS**CH_2CH_2-dinitrophenol (target protein IgE Anti-DNP), **C**ALDRWEKIRLR (target protein IgG Anti-HIV) (SEQ ID NO: 1), **C**HNTPVYKLDISEATQV (target protein IgG Anti-Ebola) (SEQ ID NO: 2), **C**HNTPVYKLDISEATQV (target protein Fab Anti-Ebola) (SEQ ID NO: 2), cyclic RGDf**C** (target protein $\alpha_V\beta_3$ Integrin), **thiolated**-streptavidin (target Biotin), and **HS**CH_2CH_2-biotin (target Streptavidin). A summary of these various binding arrangements is given in **Table 1** below, adapted from Zhang et al.[5]

Table 1.

Target	MW (kD)	Height (nm)	Probe on electrodes	K_D (nM)	Control	Peak Conductance (nS)	Binding Mode
IgE Anti-DNP	190	~5 [1]	**HS**CH_2CH_2-dinitrophenol	65	IgE isotype	0.27±0.03, 1.96±0.47	NS-S S-S
IgG Anti-HIV	150	~7 [2]	**C**ALDRWEKIRLR (SEQ ID NO: 1)	240	IgG isotype	0.33±0.03, 2.18±0.36	NS-S S-S
IgG Anti-Ebola	175	~7 [2]	**C**HNTPVYKLDISEATQV (SEQ ID NO: 2)	1400	IgG isotype	0.26±0.007, 2.09±0.09	NS-S S-S
Fab Anti-Ebola	50	~4 [3]	**C**HNTPVYKLDISEATQV	NA	IgG isotype	0.30±0.008	NS-S
$\alpha_V\beta_3$ Integrin	190	~10 [4]	Cyclic RGDf**C**	~10	a_4b_1 Integrin	0.38±0.009	NS-S
Biotin	NA	NA	**Thiolated**-streptavidin	~ 10^{-5}	NA	0.35±0.008	T-T
Streptavidin	55	~3.5 [5]	**HS**CH_2CH_2-biotin	~ 10^{-5}	NA	0.26±0.01, 1.48±0.07, 6.80±0.6	S-S

Proteins and ligands used in this study. The cysteine or thiol used for electrode attachment is in bold. Linear dimensions are from the RSCB PDB, either across a minor diameter or, for the antibodies, binding

head to binding head: [1]IgE structure 4GRG, [2]IgG, structure 4NHH, Fab fragment structure 1YUH, [4] Integrin structure 1L5G, [5]streptavidin structure 1VWA.

The bioelectronic circuit also comprises at least one protein that binds the at least one ligand. The protein can be any protein that can be expressed in a medium that allows for modification of the native protein sequence. Thus, any protein function can be incorporated into an electrical circuit so that changes induced by ligand or substrate binding, or enzyme activity can then be measured electrically.

In one embodiment, the bioelectronic circuit comprises (a) two palladium electrodes, (b) thiolated streptavidin attached to the palladium electrode, and (c) biotinylated polymerase, thereby forming an electronic contact between the electrode and the polymerase. It should be understood that while the bioelectronic circuit described below comprises palladium electrodes, thioloated streptavidin, and a biotinylated polymerase, this embodiment is illustrative of the present disclosure and the scope of the present disclosure is not limited to this one embodiment.

In one aspect of this embodiment, thiolated streptavidin with an average of 2.5 thiols per tetramer was obtained from ProteinMods (Madison, Wisconsin). The streptavidin (**31** in **FIG. 3**) was incubated in a 1 μM aqueous solution with a pair of noble metal (palladium) electrodes (the pair forming a nano-scale junction approximately 5 nm gap, **32**, **33** in **FIG. 3**). Incubation overnight produced a dense coating of the streptavidin attached to the metal electrodes by means of a surface thiol (**34**).

In order to complete the bioelectronic circuit, a bis-biotinylated ϕ29 polymerase was constructed (**35** in Fig 3). The Avitag peptide sequence, GLNDIFEAQKIEWHE (SEQ ID NO: 4), was inserted between residues G111 and K112, and a second Avitag peptide sequence was inserted between E279 and D280 in the ϕ29 polymerase sequence (the exonuclease activity of which was deleted by mutating D12 and E14 to alanine). This produced a polymerase with two Avitags separated by about 5 nm. The Avitags were biotinylated using the BirA enzyme, as is well known in the art. The resulting molecule (**35**) therefore contained two biotin molecules (**36** in **FIG. 3**). The biotinylated polymerase was incubated with the streptavidin functionalized junction for two hours (in 1 μM aqueous solution of ϕ29 polymerase). The electrical properties of the bioelectronic circuit were measured by applying a voltage bias V across it (**37**) and recording the current (**38**) through the circuit. As noted by Zhang et al., [5] this bias must be less than 100 mV to avoid contact-generated noise, and 50 mV bias was used to collect the data shown here.

To confirm that the modified polymerase was still active, rolling-circle amplification of a DNA template was carried out with the molecule **35** as bound by two streptavidin molecules. The polymerase activity,

as monitored by a DNA gel of the polymerization product (**42** in **FIG. 4**) is comparable to that of the native enzyme with exonuclease activity deleted (**41** in **FIG. 4**).

In order to confirm that the bis-biotinylated molecule **35** bridged the junction, a monobiotinylated ϕ29 polymerase was prepared. To do this, the following sequence was added to the N-terminus of the WT (but exonuclease inactivated) enzyme: MGSSHHHHHHSSGLVPRGSGLNDIFEAQKIEWHEGASS (SEQ ID NO: 5), in which five histidines are a his-tag for purification of the protein and GLNDIFEAQKIEWHE (SEQ ID NO: 4) is the Avitag.

In order to characterize the conductance of the molecular circuits in all its possible binding geometries, repeated measurements were made on different molecular junctions, and the frequency with which particular values of conductance are recorded on a logarithmic conductance scale were plotted (as described in Zhang et al.[5]). **FIG. 5** shows such distributions for the mono-biotinylated polymerase (**51**) and the bis-biotinylated polymerase (**52**). The bis-biotinylated molecule shows a new, high conductance feature (**53**) not present in the distribution measured for the mono-biotinylated molecule. This feature corresponds to about 4 nS conductance, a substantial increase over the highest conductance feature observed for the mono-biotinylated molecule (about 0.7 nS). This is a similar increase to that observed for molecules like antibodies connected by one or two specific contacts, as described by Zhang et al. [5] Note that these very high conductivities are obtained with the streptavidin molecules forming part of the circuit, showing that assemblies of several proteins in sequence maintain their quasi-metallic properties if they are properly connected.

FIG. 6 shows electrical signals obtained from a bis-biotinylated polymerase molecule in its resting state **61** and its active state **62** where it is actively polymerizing a DNA template in the presence of dNTPs and Mg. In each case, current data were recorded for about 90 s after an electrode was moved into contact with the complex. Formation of the streptavidin- ϕ29 polymerase contact takes about 20 s, after which the current (at 50 mV bias) jumps from zero to about 60 pA. In the case of the control molecule **61** the current remains fairly quiet and constant for the remainder of the run. For the active molecule **62**, bursts of noise **63** are observed, each lasting for a few seconds. A 50 ms portion of one of these bursts is also shown (**64**) - the bursts themselves consist of sub-bursts (**65**), the duration and spacing of which are consistent with the interpretation that each sub-burst (**65**) signals the incorporation of a single nucleotide (given the known kinetics of ϕ29 polymerase). This illustrates the use of the current invention in building circuits that can monitor enzyme activity electrically.

Recognizing the importance of placing a contact as close to the hydrophobic interior of a protein as possible, while still having the contact point itself appear on the surface of the protein, sites for insertion of the Avitags should be placed as close aromatic residues as possible while still exposing the site for biotinylation at the exterior of the protein. Therefore, the Avitag should be placed as close as possible to tyrosines, tryptophans or histidines that are near the surface of the protein.

In a second embodiment, the bioelectronic circuit comprises (a) two palladium electrodes, (b) thiolated biotin attached to the palladium electrode, and (c) streptavidin. This embodiment allows for a uniform coating of small molecules on the electrode.

In one aspect of this embodiment, biotinylated cystamine was used as the thiolated biotin. N,N'-bisbiotinyl-cystamine **11** (chemical structure shown in **FIG. 1**) was synthesized as described in the Examples. Because of the S-S linkage (**12** in **FIG. 1**), **11** is stable in oxidizing environments (like air). It can be reduced to the monothiol **20** (chemical shown in **FIG. 2**) in the presence of a strong reducing agent. Reducing agents are known in the art and any can be used. In some embodiments, the reducing agent is an immobilized TCEP (Tris[2-carboxyethyl] phosphine hydrochloride) disulfide reducing gel from Thermo Scientific (cat # 77712).

In this second embodiment, electrodes are functionalized with thio-biotin, and the junction is then exposed to wild type (i.e., lacking surface thiols) streptavidin. The result is a strongly conducting bridge. This is illustrated in **FIG. 7**. In a control experiment (**71**) the gap is bridged by a thiolated streptavidin molecule which has been incubated with biotin (this is because biotin binding changes the conductance of streptavidin as described by Zhang et al. [5] so it is necessary to compare biotin-bound streptavidin molecules to see the intrinsic contact differences in these experiments). In the second measurement (**72**) the electrodes are first functionalized with the mono-thiolated biotin (**20**) and then wild type streptavidin is introduced to bind and complete the circuit. The current distribution for the thiolated streptavidin (as biotin bound) directly attached to the electrodes is shown in **73**. The distribution for the wild type streptavidin captured between biotinylated electrodes is shown in **74**. A new, high conductance feature is observed at nearly 7nS (**75**) in the case where the thio-biotin molecule (**20**) is used to attach the streptavidin to the electrodes. Remarkably, connection via a specifically-binding ligand (biotin) that is, itself, attached to the electrode via a thiol, can form a better contact than a direct attachment of the protein itself via a surface thiol on the protein.

In the embodiments described above, the protein contains one contact for a ligand. In the embodiments described in the following paragraph, the protein contains a second contact for a ligand.

For example, the integrin molecule listed in **Table 1** has one binding site for a small ligand that makes an excellent electrical contact (the cyclic RGD peptide incorporating a cysteine as listed in **Table 1**). But contact to a second point cannot be made via another ligand, because this integrin only has one RGD binding site. However, a well-defined circuit can be completed by incorporating an Avitag sequence into a region near the N terminus of the integrin, so that now it has two specific binding sites: (1) the RGD peptide binding site and (2) the biotin binding site. Heterogeneous contacts like this (peptide binding at one site, biotinylation at another) have the advantage that the protein can be oriented in an assembly by exploiting the selective attachment now incorporated into the protein. For example, one electrode might be functionalized with the streptavidin, and a second with the peptide (i.e., cyclic RGD), so that the modified protein would then always be bound in a well-defined orientation with respect to both electrodes. The same technology enables sequential assembly of protein circuits. This is illustrated with the protein "AND" gate shown in **FIG. 8**.

More specifically, with protein A (**81**) designed or chosen such that binding of ligand A causes an increase in conductivity and protein B (**82**) designed or chosen such that binding of ligand B causes an increase in conductivity, wired in series, and connected between electrodes, the pair comprise a chemical AND gate because the high conductance state is only obtained when both ligand A and ligand B are present. Clearly one way to guarantee that the proteins are wired in the desired order is the use of selective contacts. Thus, the first electrode, **84**, is modified with the thio-biotin molecule **86** that binds a streptavidin molecule **87**. This in turn binds protein A **81** that has been modified so as to have incorporated two biotinylated Avitag sequences **83**. Further incubation with streptavidin places a second streptavidin **84** on protein A. At this point, protein B **82** can be bound using a biotinylated Avitag sequence **83**. If the same biotin-streptavidin coupling were to be used to complete the circuit contact to the second electrode **85**, the undesirable possibility arises that a protein A could become incorporated at the second position where a protein B is desired. In order to overcome this possibility, a heterogeneous linkage is used, in this case exploiting a peptide binding site **88** on protein B. The corresponding peptide ligand **89** bound to the second electrode **85** completes the circuit by binding to its cognate site on protein B. It will be recognized that additional useful building blocks can be created by linking one ligand to another. For example, by concatenating a peptide ligand (such as one of those shown in **Table 1**) to a biotin molecule, so that a first protein with a specific binding site to the peptide could be linked to streptavidin, for example, for subsequent incorporation into a circuit via the remaining unoccupied biotin binding sites on the streptavidin.

Another consideration that arises in connecting functional proteins into a circuit with two contact points is the possible disruption of function caused by the mechanical constraints imposed by tethering the protein

at two points. While connection at one point leaves the protein free to move much as though it were in solution, a second attachment point, especially one chosen to be distant from the first (so as to sense protein motion) could clearly disrupt protein function. Thus, it is highly desirable to incorporate a flexible linker into the region where a second contact site is incorporated. This could, of course be at either one of the two contact sites.

Sequences of amino acids that form flexible linkers are well known in the art and examples are listed on the world wide web at bmrb.wisc.edu/referenc/choufas.shtml. When selecting a particular flexible linker, there are three important design considerations.

The first consideration is that the incorporated sequence should not consist of short repeats, as this complicates cloning. To illustrate this, consider the well-known flexible linker amino acid sequence: GGSGGSGGSGGS (SEQ ID NO: 6). The corresponding DNA template is shown in **FIG. 9A**, which also shows the dimer that can be formed between primer sequences. The result, when cloned into an expression vector, is shown in **FIG. 9B** which shows the sequence of the resulting plasmid with the products of the undesired primer dimers labeled.

The second consideration is that the isoelectric point of the new protein should not be altered significantly, so that the residues in the inserted linker should be chosen to be neutral or nearly so. This is illustrated by the synthesis of a phi29 polymerase containing two Avitags for wiring into a circuit. In this case, the flexible linker was placed next to the Avitag sequence located near the N terminus. In the first case (sequence **101** in **FIG. 10**) the flexible linker (circled, **103**) has the sequence GDSTDGTSDGSS (SEQ ID NO: 7) with the result that the protein product has a calculated pI of 7.25. This small shift from the pI of the native protein (which is a pI of about 8) is enough to cause misfolding resulting in the protein appearing as a shorter product **105** in a gel **106**. When aspartic acid (D) is changed to asparagine (N) in the linker sequence (now GNSTNGTSNGSS (SEQ ID NO: 3) - **104** in sequence **102**) the native pI is restored (pI=8.13) and the product now runs at the position of the correctly folded native protein (**107** in gel **108**).

The third consideration arises when two identical sequences (i.e., two Avitag sequences) are to be inserted into the same clone expression system, as the corresponding repeated DNA sequence results in primer-dimers. To overcome this, the cloning proceeds in two steps. A clone with one Avitag sequence is first produced, and then a second clone is generated from this first clone with the second Avitag sequence inserted.

Given the flexibility of the GNSTNGTSNGSS (SEQ ID NO: 3) linker and the concern that a particular protein folding may be required to support this quasi-metallic state of the protein interior, a key question is whether the conductive properties of the protein are maintained when the linker is inserted. **FIG. 11** shows the reference conductance distribution **1101** for the two-Avitag polymerase previously shown as **52** in **FIG. 5** (lacking a flexible linker sequence). Here, the high conductance feature owing to binding the two biotinylated sites is labeled **1103**. **1102** shows the conductance distribution for the phi29 incorporating a flexible linker (protein sequence **102** in **FIG. 10**). The high conductance feature is preserved (**1104**) showing that flexible peptide linkers can be incorporated while maintaining the quasi-metallic state of the protein.

Streptavidin is tetravalent (it binds up to four biotins) so, taken together with these remarkable and unexpected electronic properties, both of the coupling scheme and of the proteins themselves, completely new avenues for building bioelectronic circuits become available.

FIG. 12 shows an "OR" gate based on the same assembly principles (the numbered components are as described for **FIG. 8**). This will enter the high conductance state if either ligand A or ligand B is present.

The tetravalent nature of streptavidin allows for even more complex circuits to be assembled. One version of this possibility is shown in **FIG. 13** where, again, numbering is as in **FIG. 8**. Here, a third protein "C" (**1300**) is attached to the junction of protein A and protein B at an unoccupied site of the coupling streptavidin **87**. If, for example, protein C is an electrochemically active protein that changes its oxidation state in the presence of oxidizing molecules, for example, then the change of charge on protein C will modulate electron transport through the A-B chain. Thus, the "A" AND "B" function can give rise to a third state that depends on the electronic state of "C".

The embodiments described thus far have all utilized both a first and a second electrode. However, the quasi-metallic conductance of protein interiors, coupled with the ligand-based linkages described here would allow for very efficient electron transfer at the surface of a sensor electrode immersed in a solution containing molecules that generate redox activity in a sensor protein (for example, the glucose sensor[1]). Thus, the protein or proteins of interest would be only linked directly to one electrode as illustrated in **FIG. 14**. Here, protein D **1400** is a redox active protein (for, example, glucose oxidase) immersed in an electrolyte **1401** containing a redox couple **1402**, **1403** (for example, oxidized and reduced glucose) with a second (remote) electrode **1404** in contact with the electrolyte **1401** also, and connected to the first electrode **84** with a means for applying a voltage bias **1405** and a means for sensing the current **1406** that flows between the first electrode **84** and the second electrode **1404**. This is an example of the use of the

linkages of the present invention as applied to improve a currently existing electrochemical sensor technology.

The bioelectronic circuit of **FIG 3** requires direct attachment of the modified coupling proteins to the electrodes. However, as shown in Zhang et al., coupling is more efficient if it is made via a small ligand attached to the electrode using an unmodified coupling protein. This has the advantage that the electrodes can be functionalized with a small ligand with which it is easier to obtain uniform coverage. Accordingly, **FIG. 15** shows a first electrode **1532** modified with the thiobiotin of **FIG. 2 1534** binding and unmodified streptavidin **1531** that in turn binds the protein **1535** that has been biotinylated at the tow sites **1536**. Similar functionalization completes the coupling to the second electrode **1533**.

The size of the gap over which protein conduction is monitored can be increased substantially at very little cost in conductivity by forming a daisy-chain of coupled proteins with each electrode as shown in **FIG. 16.** Here, a second coupling protein **1602** is added into the circuit, coupled to the first **1531** by means of a bivalent linker **1601** such as the bis-biotin shown in **FIG. 1.**

EXAMPLES

The following examples are presented for illustrative purposes and should not serve to limit the scope of the disclosed subject matter.

Sources of materials

RGD peptide (cyclo(Arg-Gly-Asp-D-Phe-Cys)) was purchased from Peptides International (Louisville, Kentucky). Peptide ligands for the anti-HIV antibody and the anti-Ebola antibody were synthesized by CPC Scientific (Sunnyvale, California) with a purity >95%. DNP and biotin disulfides were synthesized in our lab (SI Appendix Figs. S10 and S13) and reduced for 2 h before use by an immobilized TCEP (Tris[2-carboxyethyl] phosphine hydrochloride) disulfide reducing gel from Thermo Scientific (cat # 77712) following the manufacturer's instructions. Preparation of the solutions used in this disclosure is described in the SI Appendix. Anti-DNP antibody (mouse monoclonal IgE antibody), wild-type streptavidin and all other chemicals were purchased from Sigma Aldrich (Saint Louis, Missouri). Anti-HIV antibody (Anti-HIV1 p17 antibody [32/1.24.89]) and all the isotype controls were obtained from Abcam (Cambridge, MA). Anti-Ebola antibody was cultured from plants as described below. Binding affinities of all the three antibodies were measured by surface plasmon resonance (SPR). Thiolated streptavidin with an average of 2.5 thiols per tetramer was from ProteinMods (Madison, Wisconsin). Ag/AgCl reference electrodes salt-bridged by 3 M KCl or 10 mM KCl were prepared as described

previously. B. Zhang *et al.*, *Nano Futures* **1** (2017). Full details of the cyclic voltammetry are provided in the SI Appendix. The anti-Ebola antibody and the corresponding monomeric Fab fragment were prepared and purified as described in the **Example 7**.

Example 1. *Synthesis of N,N'-bis-biotinyl-cystamine **11** (chemical structure shown in **FIG. 1**).* Cystamine dihydrochloride (60 mg, 0.27 mmol) was added into DMF (2mL), followed by the addition of triethyl amine (0.44 mL, 3.19 mmol). The mixture was stirred for 30 min, to which biotin NHS ester (0.27g, 0.80 mmol) was added, and stirred at room temperature for 16 h. TLC indicted that cystamine was consumed and a product produced with an R_f value of 0.63 in 20% methanol in DCM. The mixture was co-evaporated with dichloromethane until most of DMF and TEA was removed. The residue was separated on a column with a gradient of 0-20% methanol in DCM over 120 mins at a flow rate of 3 mL/min in an automated flash chromatography instrument Teledyne Isco. The product was obtained as a white solid (0.11 g and 67.1 %). ^{1}HNMR (400 MHz, DMSO-d_6): δ 1.20-1.60 (m, 12 H), 2.07 (t, 7.6 Hz, 4H), 2.57 (d, 12.4 Hz, 2H), 2.77 (t, 6.6 Hz, 4H), 2.83 (dd, 5.1 and 12.6, 2H),3.07-3.12 (m, 2H), 3.31 (t, 6.4 Hz, 4H), 4.11-4.15 (m, 2H), 4.31 (t, 5.2 Hz, 2H); ^{13}CNMR (100 MHz, DMSO-d_6): δ 25.70, 28.50, 28.65, 35.62, 37.83, 38.37, 40.32, 55.92, 59.68, 61.52, 163.18, 172.69.

Example 2. *Synthesis of Compound 20*

Compound **20** was produced by exposing N,N'-bis-biotinyl-cystamine **11** for 2 h (immediately before use in devices) to an immobilized TCEP (Tris[2-carboxyethyl] phosphine hydrochloride) disulfide reducing gel from Thermo Scientific (cat # 77712) following the manufacturer's instructions. The product (chemical structure shown in **FIG. 2**) was dissolved in 20% methanol in DCM, the solution that was subsequently used to modify electrodes.

Example 3. *Functionalizing substrates and STM probes*

Palladium substrates for STM measurement were prepared by evaporating a 200 nm palladium film onto a silicon wafer using an electron-beam evaporator (Lesker PVD 75), with a 10 nm titanium adhesion layer. The substrates were treated with a hydrogen flame immediately before functionalizing and then immersed in solutions of thiolated DNP, biotin, streptavidin or peptides containing a cysteine residue, overnight. Substrate functionalization with small ligands was characterized by Fourier transform infrared (FTIR) spectroscopy (Fig. S9) and ellipsometry. Coverage of the substrate was monitored by STM and AFM imaging.

STM probes were etched from a 0.25mm Pd wire (California Fine Wires) by an AC electrochemical method. To avoid current leakage, probes were insulated with high-density polyethylene following the method described earlier for gold probes. M. Tuchband et al., *Rev Sci Instrum* **83**, 015102 (2012). Each probe was tested by STM in 1 mM PB buffer at +0.5 V bias to ensure the leakage current was <1pA. For functionalization, the probe was immersed in ligand solutions for 4 h or overnight. After that, it was taken out, rinsed with water, gently blown dry with nitrogen gas, and used immediately. Further details of the STM measurements are given in **Example 4**.

Example 4. *STM Measurement*

STM measurements were carried out on a PicoSPM scanning probe microscope (Agilent Technologies), coupled with a DAQ card (PCI-6821 or PCIE-7842R, National Instruments) for data acquisition. The Teflon cell, in which buffer solution and analytes were added, was cleaned with Piranha solution and then sonicated in Milli-Q water three times to remove residues (Note that Piranha solution is highly corrosive and must be handed with extreme care). In order to better control the surface potential, an Ag/AgCl reference electrode with 10 mM KCl salt bridge was connected onto the substrate. The probe was firstly engaged to 4 pA setpoint current with a bias of -0.2 V and then left to stabilize for 2 h before measurement. For STM IV sweep measurements, the servo system was first turned off and the probe was retracted by ΔZ nm with a speed of 1 nm/s. After that, the probe was suspended at that height for 1 minute, during which a custom Labview program was used to monitor the current change. Once the current exceeded a threshold of 50 pA, it was considered as a binding event and IV sweeps were started from -0.2 V to +0.2 V and then back, with a sweep rate of 1 V/s, followed by a 0.2 s resting. Subsequently, the current was checked again. If the current was still more than twice the noise level (6 pA), IV curves were continuously recorded until the bound protein molecule escaped. After one-minute of measurement, the servo system was turned on to re-engage the probe and then the whole process repeated. In each measurement, at least 1000 IV curves were collected, from which curves with overlapping up sweep and down sweep were selected (80% of the total) to construct the conductance distribution histogram. Current vs. time traces were recorded by another Labview program with a similar procedure except that the bias was held constant during the probe holding process. The analog-to-digital sampling rate is 50 KHz. Conductance measurement procedures for all the analytes were identical but with different efficiencies because of binding affinity and functionalization efficiency differences (**Table S1**).

Table S1. Frequency with which sweeps yielded an IV curve as a function of gap size and functionalization. Z_0~ 2.5 nm. No curves were obtained with control samples (**Table 1**).

Protein	Modifications		Gap size	Frequency
	Probe	Substrate		
Anti-DNP	Thio-DNP	Thio-DNP	Z_0 + 1 nm	70.1%
	Thio-DNP	Thio-DNP	Z_0 + 2 nm	46.7%
	Thio-DNP	Thio-DNP	Z_0 + 3 nm	22.8%
	Thio-DNP	Thio-DNP	Z_0 + 4 nm	9.6%
	NA	Thio-DNP	Z_0 + 2 nm	~ 0
	MCE	Thio-DNP	Z_0 + 2 nm	18.9%
Anti-HIV	Thio-peptide	Thio-peptide	Z_0 + 2 nm	42.5%
	NA	Thio-peptide	Z_0 + 2 nm	~ 0
Anti-Ebola	Thio-peptide	Thio-peptide	Z_0 + 2 nm	24.0%
	NA	Thio-peptide	Z_0 + 2 nm	~ 0
Integrin-$\alpha_v\beta_3$	RGD peptide	RGD peptide	Z_0 + 1 nm	35.9%
	RGD peptide	RGD peptide	Z_0 + 2 nm	23.7%
	RGD peptide	RGD peptide	Z_0 + 3 nm	10.5%
	NA	RGD peptide	Z_0 + 2 nm	~ 0
Thio-Streptavidin	NA	Thio-Streptavidin	Z_0	48.2%
	NA	Thio-Streptavidin	Z_0+1nm	4.3%
Anti-Ebola Fab	Thio-peptide	Thio-peptide	Z_0	54.1%
	Thio-peptide	Thio-peptide	Z_0+1nm	11.8%

Replicated measurements for each analyte were conducted with newly functionalized substrates and probes (**Table S4**). Potential relative to the reference electrode was set using a battery-powered voltage source connected between the substrate and reference electrode.

Table S4. Number of replications of each experiment

Experiment	**Times replicated**
Anti-DNP antibody	6
Anti-Ebola antibody	3
Anti-HIV antibody	3
Integrin ($\alpha_v\beta_3$)	4
SH-Streptavidin	5
SH-Biotin + Free streptavidin	2
Anti-Ebola antibody Fab	4

Example 5. *Preparation of solutions*

Thiolated DNP and biotin were prepared in freshly degassed pure ethanol to a final concentration of 0.1 mM. Peptides were firstly dissolved in degassed water as received and then aliquoted and frozen at -80 °C. Each time before use, one aliquot was taken out and diluted by degassed water to the desired concentration, usually 0.1 mM for substrate and probe functionalization. To lower the functionalizing density of DNP and peptides on the substrates, 2-mercaptoethanol (MCE) was added at 2 mM concentrations. Antibodies and isotype controls were aliquoted and stored at -80 °C and then diluted in 1 mM PB buffer (pH = 7.4) before use. For STM and solid-state chip measurements, 100 nM antibodies or isotype controls in 1mM phosphate buffer (PB) were used. Thiolated streptavidin of 1 μM in 1 mM PB buffer was prepared for substrate functionalization. Free biotin of 1 mM and wild-type streptavidin of 100 nM in 1 mM PB buffer were employed for the conductance measurements by STM. All the buffers and solutions were prepared in Milli-Q water with a conductivity of 18.2 MΩ. For all measurements, the 1 mM PB buffer (pH 7.4) was degassed with argon to avoid interference from oxygen.

Example 6. *Cyclic voltammetry*

Pd substrates were cut into 0.5 cm x 4.0 cm in size and used as the working electrode, with an active cell area of about 0.5 cm x 1.0 cm. The substrate was treated with a hydrogen flame before functionalization. Cyclic voltammetry was performed on a potentiostat (Model AFCBP1, Pine Instruments), using a Pt wire as the counter electrode and an Ag/AgCl (3M KCl) as the reference electrode. The sweep range is from -0.5 V to +0.5 V, with a sweep rate of 10 mV/s unless stated otherwise.

Example 7. *Expression and purification of anti-Ebola (EBOV) mAb 6D8 from Nicotiana benthamiana plants*

The coding sequences of heavy and light chain of 6D8 (Lai, H., *et al.*, *Plant Biotechnol J* **10**, 95-104, doi:10.1111/j.1467-7652.2011.00649.x (2012) were cloned into MagnICON-based expression vectors. Lai, H. *et al. Proc Natl Acad Sci U S A* **107**, 2419-2424, doi:10.1073/pnas.0914503107 (2010). 6D8 was then transiently expressed in *N. benthamiana* plants as described previously. Yang, M., *et al.*, *Plant Biotechnol J* **16**, 572-580, doi:10.1111/pbi.12796 (2018). 6D8 mAb was isolated and purified to > 95% homogeneity from *N. benthamiana* leaves by protein A affinity chromatography. Fulton, A. *et al.*, *J Chromatogr A* **1389**, 128-132, doi:10.1016/j.chroma.2015.02.013 (2015).

Example 8. *Generation of monomeric Fab of 6D8*

Monomeric Fab fragments were prepared from 6D8 by using the Pierce Fab Preparation Kit (Thermo Scientific) according to the manufacturer's instructions (Thermos Scientific Pub. No. MAN0011651).

Briefly, purified 6D8 was first incubated with papain immobilized to agarose beads at 37ºC for 6-12 hr. The digested mAb mixture was then recovered by centrifugation at 5000xg for 1 min and separated on a protein A chromatography column. The Fab fragment was recovered in the flow through fraction, while the Fc fragment and undigested mAb were trapped in the protein A column. The successful production of monomeric Fab was verified by SDS-PAGE analysis under both reducing and non-reducing conditions.

Single-Molecule Conductance Measurements

Reproducible two-point measurements of the conductance of molecules require reproducible contacts, (7) so the reproducible observation of large (nS scale) conductance fluctuations in single integrin molecules (bound to just one of two electrodes by their cognate ligands) was a surprising finding. (8) This prior work did not probe the low bias region (where fluctuations were absent) owing to leakage currents that obscured any DC current through the protein. Here, a scanning tunneling microscope (STM) was used to make single-molecule measurements in solution, systematically exploring the role of contacts, both specific and non-specific. With suitably insulated STM probes, (9) and potential control of the electrodes the background leakage current was reduced to less than 1pA over the entire bias range. With adequate stabilization, the STM gap remained constant over periods of a minute so it was possible to disable the gap control servo, retract the tip, and record current-voltage (IV) curves. Up to 60 such curves (sweeping both up and down) were recorded before re-engaging the servo and repeating the process on another area of the substrate. In order to make two specific contacts, bivalent antibodies (an IgE and two IgG's) were used, each of which presents two binding sites, as well as streptavidin which binds up to four biotin molecules, so that epitope- or biotin-functionalized electrodes could be bridged by specific bonds. In the cases where bare metal electrodes were used, contacts were made to surface thiols on streptavidin modified with an average of 2.5 surface thiols per molecule. In addition, measurements were repeated using integrin, which can form a specific bond with only one of the two peptide-functionalized electrodes. Proteins and ligands are listed in **Table 1**.

Measured Conductances Depend On Contacts

Currents were only observed when the protein was bound specifically to at least one of the two electrodes Typically, no current was recorded for several seconds after retraction, after which the current jumped to a large (and variable) value in the presence of bound protein. Although the current fluctuates over minute time-scales, it is usually stable over a few seconds, so that 80% of the recorded curves on the sweep up are reproduced on the sweep down (data not shown). Controls (buffer alone or non-cognate proteins in solution) gave no signals. The rapidly fluctuating (ms-timescale) telegraph noise (TN)

reported (8) for integrin was also observed for anti-DNP (data not shown), and all the other proteins studied above 0.1V. It is a ubiquitous signal of protein capture, showing the same two-level switching in all cases. These fluctuations were originally observed for a protein captured in a fixed-junction chip (8) and though the present work uses an STM, measurements of TN were replicated for one of the proteins studied here (anti-DNP) in a chip as well as in the STM to show that these are not some artifact of the measurement method (data not shown). The voltage threshold for TN does not depend on gap until the contact is almost broken (data not shown) implying that it is associated with fluctuations of the contacts driven by a potential drop that occurs mostly at the contacts, as previously proposed (8) and discussed in more detail below.

With the exception of the TN, the response is linear, so that each IV trace can be characterized by a single conductance value, G. Measured distributions of G follow the log-normal distribution usually observed in single molecule measurements (data not shown). (10) The distributions are similar to distributions of current values obtained by recording current vs. time at a fixed gap and bias (data not shown) so the distribution was ascribed to different kinds of contact between the electrodes and the molecule. The distributions for integrin (gap = 4.5 nm) and thio- streptavidin (gap = 2.5 nm) have a single peak at about 0.3 nS (data not shown). Bare metal electrodes were used to capture the thiolated streptavidin, where the thiol mediated contacts displace the contamination on the electrode surfaces, (11) forming direct metal-molecule contacts. The integrin was captured by the cyclic RGD peptide at only one of the two electrodes, and no signals were observed unless both electrodes were functionalized. Functionalization with peptides allows for non-specific contacts with hydrophilic sites on the protein at the electrode that is not specifically coupled. The three antibodies yielded two conductance peaks (~0.3 nS and ~2 nS), suggesting two binding modes: NS-S as for the integrin, and the desired S-S when both antigen-binding sites bind specifically (data not shown). This interpretation was tested by replacing the peptides on one electrode with mercaptoethanol, making it hydrophilic and capable of forming an NS-S bridge. Only a single peak was observed (data not shown). As a further test, a Fab fragment from the anti-Ebola IgG was prepared with only a monovalent binding head. The fragment was too small to bridge the 4.5 nm gap, so the data were recorded in a 2.5 nm gap. There is only a single peak in the conductance distribution, reflecting the single NS-S contact (data not shown). Thus, the higher conductance peak must correspond to conduction via the two antigen binding sites. In order for this effect to be seen, the data set must be dominated by single molecule contacts. It is striking that the conductance of a single Fab fragment across a 2.5 nm gap is much smaller than the conductance of an antibody across a 4.5 nm gap (data not shown). This suggests that the intrinsic internal conductances of the proteins are much higher than the measured

(contact-limited) values. This finding accounts for the previous reports of similar conductances measured for proteins of very different sizes. (12, 13)

Conductances Do Not Depend On Gap Size

The existence of an internal (through-molecule (14)) high-conductance path is illustrated by a series of measurements taken at different gap sizes, using the technique described above, but increasing the amount of the initial tip retraction (data not shown). Strikingly, the peak conductance values do not change with the gap size though the frequency with which data are accumulated falls (data not shown). This effect reflects the area of the probe available for contacts at a given height. Very few sites are available when the gap is comparable to the protein height (listed for similar structures found in the protein data base in **Table 1**). Gap-independent conductance has been reported before for azurin (see the SI of Ruiz et al. (15)) and a rod-like molecule trapped between a probe and a substrate. (14) As pointed out above, the contact point changes over the (~minute) course of a measurement, a reflection of the angstrom-scale change in the position of the STM probe. It is these various contact geometries that generate the overall shape of the conductance distributions (data not shown). Since the distributions retain the same peak positions and shapes at the different gap sizes, the data show no indications of proteins being “squeezed” at the smaller gap sizes.

Plots of the voltage thresholds for the turn on of TN as a function of gap size show that the voltage thresholds do not change significantly with the gap size. Thus, TN fluctuations must be driven by the local field at the metal-molecule interface, with relatively little potential dropped across the interior of the protein. This is also consistent with our finding that the lifetime of the TN is exponentially related to the peak current value, an observation that can be accounted for by a single "weak link" tunneling junction in the circuit that dominates the conductance. (8)

Conductance Is Sensitive To Changes Of Protein Structure

Since the conductance path follows the protein geometry, either internally, or along a surface contour, changes in protein geometry, and thus in the conduction path, could affect which contact points control the conductance. This would enable direct electrical sensing of structural changes of the proteinThis effect is demonstrated in **FIG. 7A**. Biotin complexation in a thiolated-streptavidin sample changes the conductance distribution significantly (**FIG. 7A**). A streptavidin molecule has four biotin binding sites, (16) so that the unthiolated apo-protein can be crosslinked by two biotins. A thiolated biotin was synthesized and functionalized both the probe and substrate with it (**FIG. 7B**), subsequently flowing apo-streptavidin into the sample cell. The consequent G distribution had three peaks, with the highest at

nearly 7 nS. Thus, the measured conductance is sensitive to both local changes in protein structure and the chemical nature of the contact, showing how contacts are affected by structural changes of the protein. Thiolated streptavidin was attached to the substrate and probed with a biotinylated probe with similar results (data not shown) demonstrating that a single biotin-mediated contact is sufficient to generate the high conductance state at ~ 7nS.

Possible Mechanisms

Electron tunneling decays far too rapidly to account for long-range transport. A tunneling conductance can be estimated from $G \sim G_0 \exp(-\beta x)$ where G_0 is 77μS and $\beta \sim 1 Å^{-1}$. (24) For a small protein with x~4 nm, this yields $G < 10^{-21}$ S, 12 orders of magnitude smaller than observed. To account for the observation of nS conductance over 10 nm distances would require a $\beta < 0.1 Å^{-1}$. In the well-studied case of DNA, thermally-activated hopping (17) leads to nearly-distance independent transport when the distance between the readily-ionized guanines exceeds three nucleotides. (18) Similar transport (via readily oxidized amino acids) has been observed in peptides. (19) In these cases, transport was limited by charge injection, and the ~1.5 eV barrier to charge injection was overcome using a chromophore excited with 630 nm (~2eV) light. If a similar transport mechanism operates in the case of charge injection from electrodes, the barrier would be determined by the energy gap between the Fermi energy of Pd (work function 5.2 eV) and the absolute redox potential of the readily oxidized residues tyrosine and tryptophan. These potentials are ~+1 to + 1.2 V vs. NHE (20) (21) so using 4.4 eV for the work function of the NHE (22) gives absolute potentials around 5.4-5.6 eV below the vacuum or a barrier of +0.2 to 0.4 eV with respect to the Fermi energy of Pd. Thus, a barrier of this magnitude must be overcome by the bond polarization associated with the binding of the protein to the electrode. This is well within the range of work-function changes observed for small molecules attached to a noble-metal surface by a thiol linkage. (23) Significant currents were obtained in three situations: (a) when contacts to both electrodes are via direct, thiol-mediated bonding; (b) when one bond is formed by specific binding to an epitope or ligand and another is formed by non-specific interactions between hydrophilic molecules attached to the electrode and the hydrophilic exterior of a protein and (c) the largest currents are observed when the protein is bound by recognition ligands at both electrodes (the ligand being linked via thiols to the electrodes in all cases). Weak, non-specific bonds do not result in significant current flow: at least one attachment must be via a covalent or ligand-mediated linkage. Thus, the barrier to charge injection is overcome by the binding of at least one specific ligand if the protein itself is not covalently modified to bind directly to the electrode.

Small changes in interfacial charge at a contact have been shown to affect transport strongly (24) and this is a variable we can change using potential control, albeit only over a small range if Faradaic currents are to be avoided. Presumably, small changes are dominated by a much larger field owing to bond polarization at the interface. In addition to the redox potentials of amino acid residues, the three-dimensional folding of the protein must play an important role. This is because small peptides that are stretched in a break junction do not conduct (25) whereas similar small peptides, folded on an electrode surface, do conduct. (26) This could be a consequence of some special geometry (27) or arrangement of hydrogen bonds. (28)

We turn finally to the fluctuations that set in above ±100 mV applied bias. The small dependence of this threshold voltage on gap size (data not shown) is consistent with the hypothesis that the internal conductance of the proteins is much higher than the conductance at the contacts, implying that these signals arise from voltage-driven fluctuations of the contacts themselves. We proposed such a mechanism in our earlier study of integrin (8) where we showed that the lifetime of the "on" states, τ, was related to the peak current, i_p, of the telegraph noise peaks via $\tau \propto \ln(i_p)$, a relationship that can be explained by means of a single barrier determining both current and bonding strength. Once turned on, the current grows linearly with voltage, indicating that a new Ohmic conductance channel opens (data not shown). The turn-on process is described by an exponential of the form $exp\left(\frac{|V|-V_c}{kT/e}\right)$ where V_c is an activation voltage. Fits yield V_c ~ 0.25V, a value characteristic of hydrogen bond strengths in water (29) suggesting that a hydrogen bond may be the 'weak link' in the circuit.

It is interesting to note that this 0.25V barrier is similar to the charge injection barrier deduced from the redox potentials of the amino acids, as discussed above. If the charge injection rate was limited by thermally activated hopping over a 0.22 to 0.47V barrier, and it is this rate that determines the conductance, then we would expect to observe a conductance of $\approx G_0 exp\left(-\frac{V}{ekT_B}\right)$ where 0.22 <V< 0.47 volts, yielding from 12 nS to 0.5 pS, a range which encompasses the values reported here.

Role Of Specific Binding In Electronic Conductance

We conclude that specific ligand-receptor interactions form good electrical connections to proteins. This is illustrated by the data shown in **FIG. 7A** and **FIG. 7B**. Connections made via covalent (thiol) modification of surface lysines directly bonded to the metal electrodes yield a lower maximum conductance (0.56 nS) than the non-covalent streptavidin-biotin coupling linked to the electrodes via a thiol-terminated ethane linkage (6.8 nS). This conductance is barely altered when only one biotinylated

linker is used (data not shown). Thus, a weaker coupling to the hydrophobic interior of a protein is more effective that a stronger coupling to the hydrophilic exterior, even if only one such coupling is made. If, once injected, electrons move readily in the interior of the protein, then a second (non-specific) contact will act only as a barrier at the hydrophilic surface of the protein. Such a mechanism would account for the high conductance of integrin when bound by a ligand at only one site, and also for the complete lack of conductance when both contacts are non-covalent and non-specific.

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It will be apparent to those in the art that specific embodiments of the present disclosure may be directed to one or more of the above- and below-indicated embodiments in any combination.

While particular materials, formulations, operational sequences, process parameters, and end products have been set forth to describe and exemplify this invention, they are not intended to be limiting. Rather, it should be noted by those ordinarily skilled in the art that the written disclosures are exemplary only and that various other alternatives, adaptations, and modifications may be made within the scope of the present disclosure. Accordingly, the present disclosure is not limited to the specific embodiments illustrated herein but is limited only by the following claims.

What Is Claimed Is:

1. A bioelectronic circuit comprising:

(a) a first electrode and a second electrode,

(b) a first ligand and a second ligand that are specific for a protein, wherein the first and second ligands are modified so that the first ligand attaches to the first electrode and the second ligand attaches to the second electrode, and

(c) a protein that is modified to bind the first and second ligands, wherein the binding of the protein to the first and second ligands forms an electronic contact between the electrode and the protein,

wherein the protein comprises at least one protein selected from the group consisting of

i) IgE Anti-DNP, IgG Anti-HIV, IgG Anti-Ebola, Fab Anti-Ebola, $\alpha_V\beta_3$ Integrin, and streptavidin, or
ii) a polymerase, endonuclease, helicase and a proteosome protein.

2. The bioelectronic circuit according to claim 1, wherein the electrode comprises a metal selected from the group consisting of palladium, platinum or gold.

3. The bioelectronic circuit according to claim 1 or claim 2, wherein the first and/or second ligand is selected from the group consisting of $HSCH_2CH_2$-dinitrophenol, CALDRWEKIRLR (SEQ ID NO: 1), CHNTPVYKLDISEATQV (SEQ ID NO: 2), cyclic RGDfC, thiolated-streptavidin, and $HSCH_2CH_2$-biotin.

4. The bioelectronic circuit according to claim 1, wherein the first and/or second ligand is modified to comprise a thiol, amine, disulfide or cyanide moiety.

5. The bioelectronic circuit of claim 1, wherein the first and/or second ligand is a thiolated streptavidin, and/or wherein the protein is modified to contain biotin.

6. The bioelectronic circuit of claim 1, wherein the protein is bis-biotinylated polymerase.

7. A bioelectronic circuit comprising:

(a) a first electrode and a second electrode,

(b) a first ligand that is specific for a protein, wherein the first ligand is modified so that it attaches to the first electrode,

(c) a protein that binds the first ligand and is modified to bind a second ligand, and

(d) a second ligand that binds the protein and is modified so that it attaches to the second electrode, thereby forming an electronic contact between the first and second electrodes and the protein,

wherein the protein comprises at least one protein selected from the group consisting of

i) IgE Anti-DNP, IgG Anti-HIV, IgG Anti-Ebola, Fab Anti-Ebola, $\alpha_V\beta_3$ Integrin, and streptavidin, or

ii) a polymerase, endonuclease, helicase and a proteosome protein.

8. The bioelectronic circuit of claim 7, wherein the binding site on the protein that is bound to the first ligand is in proximity to a flexible linker sequence, and/or wherein the second binding site on the protein that is bound to the second ligand is in proximity to a flexible linker sequence.

9. The bioelectronic circuit of claim 8, wherein the flexible linker sequence comprises GNSTNGTSNGSS (SEQ ID NO: 3).

10. A method for detecting the activity of a polymerase, the method comprising introducing a solution of DNA template and nucleotriphosphates to the bioelectronic circuit of claim 1, wherein polymerized product indicates that the polymerase is active.

11. A method for detecting the activity of protein, the method comprising introducing a substrate of the protein to the bioelectronic circuit of claim 1 and detecting electrical changes.

12. A system for electrical measurement of protein activity comprising:

(a) a bioelectronic circuit of claim 1,

(b) a means for applying a bias between the first and second electrode, and

(c) a means for detecting the current through the bioelectronic circuit.

13. A bioelectronic circuit comprising:

(a) a first electrode and a second electrode,

(b) a first ligand and a second ligand that are specific for a protein, wherein the first and second ligands are modified so that the first ligand attaches to the first electrode and the second ligand attaches to the second electrode,

(c) a first protein and a second protein that bind the first ligand and the second ligand, and

(d) a third protein that is modified to bind the first and the second proteins, wherein the binding of the third protein to the first and the second proteins forms an electronic contact between the electrode and the protein.

15. The bioelectronic circuit of claim 13, wherein the first and second proteins are streptavidin.

16. The bioelectronic circuit of claim 13, wherein the first and second ligands are biotin.

17. The bioelectronic circuit of claim 13, wherein the third protein is biotinylated.

18. A bioelectronic circuit comprising:

(a) a first electrode and a second electrode,

(b) a first ligand and a second ligand that are specific for a protein, wherein the first and second ligands are modified so that the first ligand attaches to the first electrode and the second ligand attaches to the second electrode, and

(c) at least a first protein and a second protein that bind the first ligand and the second ligand, and may be coupled to further proteins via ligands, extending the range over which conduction is obtained.