In Situ Engineering of Intracellular Hemoglobin for Implantable High-Performance Biofuel Cells

Huilfeng Chen*, Zhengyu Bai*, Xinqi Dai, Xiaqiao Zeng, Zachary P. Cano, Xiaoxiao Xie, Mingyu Zhao, Matthew Li, He Wang, Zhongwei Chen, Lin Yang,* and Jun Lu*

Abstract: The key challenge for the broad application of implantable biofuel cells (BFCs) is to achieve inorganic–organic composite biocompatibility while improving the activity and selectivity of the catalysts. We have fabricated nanoengineered red blood cells (NERBCs) by an environmentally friendly method by using red blood cells as the raw material and hemoglobin (Hb) embedded with ultrasmall hydroxyapatite (HAP, Ca_{10}(PO_{4})_{6}(OH)_{2}) as the functional BFC cathode material. The NERBCs showed greatly enhanced cell performance with high electrocatalytic activity, stability, and selectivity. The NERBCs maintained the original biological properties of the natural cell, while enhancing the catalytic oxygen reduction reaction (ORR) through the interaction between –OH groups in HAP and the Hb in RBCs. They also enabled direct electron transportation, eliminating the need for an electron-transfer mediator, and showed catalytic inactivity for glucose oxidation, thus potentially enabling the development of separator-free BFCs.

Enzyme biofuel cells (EBFCs)[1] have attracted considerable attention as micro,[2] or even nanoscale power[3] sources for implantable biomedical devices, such as cardiac pacemakers,[4] implantable self-powered sensors,[5] and biosensors for monitoring physiological parameters.[6] There are two types of EBFCs, which differ in the operating mechanism; namely, mediated electron transfer (MET)[7] and direct electron transfer (DET).[8] EBFCs. MET EBFCs rely heavily on redox mediators to shuttle electrons between biocatalytic active sites and electrode surfaces, whereas DET EBFCs enable electron transfer from the enzyme active sites directly to the electrode.[9] In either case, the other electrode (cathode) requires an oxygen-reduction catalyst, such as laccase[10] or bilirubin oxide (BOD).[11] to facilitate the oxygen reduction reaction (ORR). Unfortunately, in general, the current catalysts under investigation show very low catalytic performance as well as poor adhesion to the electrode surface. Such drawbacks drastically limit the practical application of EBFCs.[12]

Although approaches such as electrode nanomodification,[13] enzyme immobilization,[14] and redox-mediator addition[15] have been intensively investigated to facilitate electron transfer between enzymes and electrodes, they usually face some challenges, such as avoiding the deformation and inactivation of enzymes,[16] preventing nanotoxicity,[17] or improving the stability of the cell.[18] As is well-known, the biosafety of nanomaterials synthesized in vitro for long-term operation in the body, in other words, the use of invasive, external, and foreign (relative to the nature of the cell) nanomaterials, is controversial, for many reasons, including their unexpected migration and accumulation.[19] Nanobioengineering,[19] which has already seen great success in the fields of medicine, agriculture, environment, and electronic systems offers a potential opportunity to tune the biofunctionality of materials with good biocompatibility.

Red blood cells (RBCs), which make up nearly 40–45% of blood volume, are responsible for oxygen transport to each body tissue. The hemoglobin (Hb) in RBCs consists of two α- and two β-globins encapsulated by a phospholipid bilayer membrane, each of which has a single heme unit as a prosthetic group. Hb is the iron-containing oxygen-transport metalloprotein, which brings oxygen from the lungs or gills to the rest of the body. Owing to this unique function, RBCs are widely employed in electronic systems, such as biosensors, biological monitoring systems,[21] and bioelectrocatalysts.[22] We demonstrate herein the construction of nanoengineered red blood cells (NERBCs) by using the intracellular bonds between the exogenous Ca³⁺ and PO₄³⁻ to generate extremely small hydroxyapatite (Ca_{10}(PO_{4})_{6}(OH)_{2}, HAP) particles in situ. The generated NERBCs contain built-in HAP nanodots with a size of approximately 3.1 nm that exhibit strong interaction with Hb owing to the anchored and secured nature of the HAP nanodots, which prevents the

Collaborative Innovation Center of Henan Province for Green Manufacturing of Fine Chemicals, Key Laboratory of Green Chemical Media and Reactions, Ministry of Education, School of Chemistry and Chemical Engineering and College of Physics and Materials Science, Henan Normal University
Xinxiang, Henan 453007 (P. R. China)
E-mail: baizhengyu@htu.cn
yanglin@htu.edu.cn
X. Zeng, M. Li, J. Lu
Chemical Sciences and Engineering Division
Argonne National Laboratory
Lemont, IL (USA)
E-mail: julu@anl.gov
Z. P. Cano, M. Li, Z. Chen
Department of Chemical Engineering, Waterloo Institute for Nanotechnology, Waterloo Institute for Sustainable Energy
University of Waterloo
200 University Avenue West, Waterloo, ON N2L 3G1 (Canada)
[*] H.C. and Z.B. contributed equally to this work and should be considered co-first authors.
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migration and unexpected accumulation of nanodots. Moreover, the overall morphology and biological properties of natural red blood cells, including membrane permeability and longevity, are well preserved. Furthermore, the NERBCs have stronger oxygen-adsorption capacity and show better performance than natural red blood cells, as supported by both experimental results and theoretical calculations. We demonstrate herein that when they are employed as a cathode catalyst for BFCs, they show clear advantages, including increased current density, a long lifetime, high selectivity, and excellent biocompatibility.

The in situ synthesis of NERBCs consisted of two simple processes. Briefly, Ca\textsuperscript{2+} was first added to a solution of RBCs, and the mixture was stirred gently for 1.5 h at 4°C. This step is important to allow the Ca\textsuperscript{2+} to pass through the cell membrane and effectively reach the interior of RBCs, thus enabling it to combine with Hb. Subsequently, the solution was centrifuged, and the resulting precipitate was washed. In the second step, phosphate buffer solution (PBS, pH 9) was added to the above system, and the mixture was incubated for 2 h at 4°C, during which PO\textsubscript{4}\textsuperscript{3–} and OH\textsuperscript{–} passed through the RBC cell membrane and entered the cells, thus leading to the generation of the NERBCs.

Scanning electron microscopy (SEM) analysis indicated that the cell morphology was not changed after the synthesis of NERBCs (Figure 1a,b), thus suggesting that the nanodot formation process did not harm the RBCs. X-ray diffraction (XRD) indicated that the as-synthesized nanoparticles separated from NERBCs were HAP crystals with a hexagonal lattice (Figure 1c), and transmission electron microscopy (TEM) clearly showed that the intracellular HAP nanodots were polycrystalline (Figure 1d, inset). Individual intracellular HAP nanodots showed lattice fringe spacing of 0.342 and 0.339 nm (Figure 1c); these values are in good agreement with the (002) plane of the HAP crystals. The intracellular HAP nanodots were homogeneously dispersed with a uniform size of (3.1 ± 0.1) nm (Figure 1f). Such extremely small nanodots and uniform dispersion are difficult to achieve by using other chemical methods and most likely indicate a high acceptance of the nanodots into the RBC structure. These results not only indicate that nanocrystalline HAP was produced in the cells, but also that the generated HAP had excellent biocompatibility.

To determine the interaction between intracellular Hb/\textsuperscript{Ca}\textsuperscript{2+} and HAP nanodots after the first and second steps of the synthesis, we analyzed the FTIR spectra (see Figure S1a in the Supporting Information) of pure Hb obtained by hemolysis of native RBCs, Ca\textsuperscript{2+}/Hb separated from Ca\textsuperscript{2+}/RBCs, and intracellular HAP/Hb separated from NERBCs. As compared with the FTIR spectrum of pure Hb, in the spectrum of Ca\textsuperscript{2+}/Hb, the amide I peak position shifted from 1638 to 1646 cm\textsuperscript{-1}, and the amide II peak shifted from 1523 to 1537 cm\textsuperscript{-1}, thus indicating a strong interaction between Ca\textsuperscript{2+} and Hb, which lays the foundation for generating HAP. For HAP-Hb, the amide I and amide II stretch vibrations both shifted to higher wave numbers (1650 and 1540 cm\textsuperscript{-1}), thus indicating that HAP nanodots combined with Hb. Moreover, the vibrational bands at 567, 604, and 1031–1120 cm\textsuperscript{-1} could be assigned to O–P–O antisymmetric bending \nu\textsubscript{as}, O–P–O bending \nu\textsubscript{a}, and P–O bending \nu\textsubscript{p} respectively. These bands substantiate the existence of HAP, in accordance with the XRD pattern. Thermogravimetric analysis (see Figure S1b) was performed under an air atmosphere from 20 to 750°C to detect the content of HAP nanodots inside NERBCs. The major mass losses of NERBCs and native RBCs were observed at 220–550°C because of the combustion of RBCs. After 550°C, RBCs were burned out, and the remaining amount of intracellular HAP nanodots was about 5% of the original weight. In short, this characterization confirmed that HAP nanodots were successfully generated and combined with Hb inside the RBCs.

The formation of HAP nanodots inside the RBCs could be identified by means of intracellular staining of HAP nanodots with tetracycline hydrochloride (Figure 2). Tetracycline hydrochloride can couple with Ca\textsuperscript{2+} to generate green fluorescence under UV light (360–370 nm), thus acting as a fluorescent probe for HAP nanodots. The z-axis focal plane of confocal laser scanning microscopy (CLSM) images of NERBCs showed that the green fluorescence followed a pattern of shallow–deep–shallow as the confocal plane changed in the z-direction (Figure 2a–i). This trend indicates that HAP nanodots are synthesized inside NERBCs. As a control, there was no fluorescence signal in native RBCs.
(see Figure S2), thus manifesting the absence of HAP nanodots in native RBCs. These results indicate that the NERBCs were successfully constructed. 2D mapping of the fluorescence signal of selected NERBCs (Figure 2j) further confirmed that the synthesized HAP nanodots were inside the NERBCs.

Optical images of native RBCs and NERBCs (see Figure S3a,b) showed that the macroscale morphology of NERBCs was exactly the same as that of the native RBCs, and no aggregation was observed. Furthermore, both kinds of cells exhibited good dispersion, although the NERBCs were slightly darker on account of the presence of intracellular HAP nanodots. An osmotic fragility test was carried out to determine the integrity of the cell membrane. The osmotic fragility curve of the NERBCs was nearly identical to that of the native RBCs (Figure 3a), thus indicating that the two kinds of cells shared similar rupture profiles.

Figure 3. Physical properties of native RBCs and NERBCs. a) Osmotic fragility curves. b) Hemolysis of NERBCs and native RBCs as a control. c) Part of the oxygen equilibrium curves of NERBCs and native RBCs.

Electrochemical tests revealed that the NERBCs possessed DET activity and higher electrocatalytic oxygen reduction ability. A pair of well-defined redox peaks were observed at around 0.62 and 0.7 V (vs. RHE) in N$_2$-saturated PBS solution containing 4% (v/v) NERBCs (Figure 4a). These Faradaic currents could be due to the Fe$^{II}$/Fe$^{III}$ redox couple in Hb molecules. The formal potential of the Fe$^{II}$/Fe$^{III}$ couple of hemoglobin in RBCs was estimated to be 0.66 V (vs. RHE), taken at the midpoint of the redox peaks. These results show that hemoglobin molecules in NERBCs demonstrate DET activity on the bare carbon cloth (CC) electrode.

Cyclic voltammetry (CV) measurements of the electrocatalytic ORR activity of native RBCs and NERBCs were carried out in O$_2$-saturated PBS solution containing 4% (v/v) of each cell type. Both NERBCs and native RBCs showed an electrochemical reduction response because of abundant O$_2$ in the PBS solution (Figure 4b). As compared with native RBCs, the reduction peak current of NERBCs increased by about 40%, from 1.92 to 2.67 mA cm$^{-2}$. The onset potential of NERBCs was about 0.47 V (Figure 4c), whereas that of native RBCs is about 0.45 V. These data clearly indicate that the NERBCs possess higher electrocatalytic oxygen reduction ability.

CV curves were recorded with different sweep rates from 0.01 to 1 V s$^{-1}$ in the PBS solution containing 4% NERBCs (see Figure S4a), and the peak currents were determined by subtracting the non-Faradaic current values. The peak currents were plotted against the sweep rate (see Figure S4b). Since the peak current of CV is proportional to the sweep rate in a surface-controlled process, the observed linear relationship indicates that the bioelectrocatalytic ORR is a surface-controlled process. Furthermore, NERBCs showed much better stability for ORR than RBCs under the same conditions. Figure 4d displays the open-circuit-potential-time (OCP-t) curves. The potential of the half-cell with NERBCs reached a maximum of 0.53 V and continued to rise slowly up to 84 h after initial assembly. In comparison, native RBCs reached a maximum OCP of only 0.42 V, and up to 84 h the OCP value even declined slowly. These results indicate that although RBCs possess stable electrochemical properties, the stability of NERBCs is much better than that of

hemolysis in fresh autologous serum (Figure 3b), thus demonstrating no evident harmful effect of the synthesis process on the proteins of the membranes after the in situ formation of intracellular HAP nanodots. Hence, our in situ synthesis is mild enough to avoid damage or cause any noticeable change in comparison to the original RBCs, which suggests that the created NERBCs have excellent biocompatibility.

To explore the effect of intracellular HAP nanodots on the oxygen-adsorption capacity of NERBCs, we obtained oxygen equilibrium curves by using a Hemox-Analyzer (TCS Scientific, CA). As compared to native RBCs ($P_{50} = (25.51 \pm 0.56)$ mm Hg), a clear left shift was observed for the NERBCs ($P_{50} = (17.84 \pm 0.63)$ mm Hg; Figure 3c; see also Figure S3c), thus indicating that the Hb oxygen affinity of NERBCs is much higher than that of native RBCs. This phenomenon may be induced by the Bohr effect, which dictates that as the pH value increases, the affinity of Hb for oxygen increases, causing the oxygen curve to shift to the left.

To explore the effect of intracellular HAP nanodots on the RBCs, we obtained oxygen equilibrium curves by using a Hemox-Analyzer (TCS Scientific, CA). As compared to native RBCs ($P_{50} = (25.51 \pm 0.56)$ mm Hg), a clear left shift was observed for the NERBCs ($P_{50} = (17.84 \pm 0.63)$ mm Hg; Figure 3c; see also Figure S3c), thus indicating that the Hb oxygen affinity of NERBCs is much higher than that of native RBCs. This phenomenon may be induced by the Bohr effect, which dictates that as the pH value increases, the affinity of Hb for oxygen increases, causing the oxygen curve to shift to the left.

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native RBCs. NERBCs had no evident effect on glucose in
N$_2$-saturated PBS solution (Figure 4c), which confirms that
NERBCs can selectively catalyze ORR.

Subsequently, we simulated the components in the blood
to conduct a biological battery test. The performance of a
membraneless single cell with an O$_2$–RBC | CC biocathode
construction (Scheme 1) was measured. The anode was made
of glucose as the raw material, and the concentration of the
glucose solution used was equal to that of glucose in human
blood to ensure relevance and comparability. The observed
open-circuit voltage (OCV) of the NERBCs was 0.636 V, and
the maximum power density attained was 21.11 mW cm$^{-2}$
(Figure 4f), which are higher than the OCV and power
density of native RBCs. The $I$–$V$ curve and power density
confirm that the performance of the BFC equipped with
NERBCs is approximately 1.5 times that of native RBCs.

These results indicate that the electrocatalytic ORR perfor-

tometry curves and oxygen equilibrium curves of native
RBCs and CaHPO$_4$@RBCs were obtained. The bioelectro-
catalytic oxygen reduction capability of the CaHPO$_4$@RBCs
was similar to that of the native RBCs (see Figure S6a).
Furthermore, as compared with native RBCs ($P_{\text{O}_2} = (25.51 \pm 0.56)$ mm Hg), there was a slight left shift of the oxygen curve of CaHPO$_4$@RBCs ($P_{\text{O}_2} = (23.37 \pm 0.63)$ mm Hg) (see Figure S6b). These results indicate that the hemoglobin oxygen
affinity of CaHPO$_4$@RBCs was in fact almost the same as that
of natives RBCs. Taken together, these results suggest that the
difference in the electrocatalytic activity between CaHPO$_4$@RBCs and NERBCs may be due to the absence/
presence of –OH groups.

To further explore the origin of the high electrocatalytic
activity of NERBCs, we performed DFT calculations using the (002) surface model of HAP, which was the most stable
HAP surface obtained according to XRD and TEM measure-
ments (see the Supporting Information for computational
details). We calculated the potential-energy ($E$) surface of
the system as the distance ($d_{\text{C-O}}$) between the carbon atom (see Figure S7a) in Hb and –OH on the HAP surface decreased
from 3.35 to 1.46 Å (see Figure S7d) and found a value of
$-0.33$ eV for this process, thus indicating that the transfer of
–OH from the center of HAP (002) to the side face of Hb is
thermodynamically favored. This transfer enables a strong
interaction between the iron center in Hb and oxygen in the
presence of the adsorbed –OH, thus weakening the O–O
bond and facilitating the O$_2$ reduction process.

We examined the most stable configuration for Hb
adsorption on the HAP (002) surface (see Figure S8a) and
the most stable structure for O$_2$ adsorption on Hb (see Figure S8b). In the latter, the nearest bonding distance
between iron and oxygen ($d_{\text{Fe-O}}$) was found to be 1.78 Å.

Figure 4. Oxygen reduction reaction electrochemical measurement. a) CV curves of bare CC electrodes in N$_2$-
saturated PBS (2 x, pH 7.4) solution and in N$_2$-saturated PBS solution containing 4 % NERBCs (v/v), with
a sweep rate of 50 mV s$^{-1}$. b) CV (scan rate: 50 mV s$^{-1}$) and c) linear sweep voltammetry (LSV) curves (scan
rate: 5 mV s$^{-1}$) of NERBCs and native RBCs in O$_2$-saturated PBS solution. d) OCP–$V$ curves of NERBCs and
native RBCs acquired under open-circuit conditions in a half-cell. e) CV curves of CC electrodes in N$_2$-saturated
PBS solution containing 4.5 mm glucose, N$_2$-saturated PBS solution containing 4% (v/v) NERBCs, and N$_2$-
saturated PBS solution containing 4.5 mm glucose and 4 % NERBCs (v/v). f) $I$–$V$ and power characteristics of
a single cell composed of a O$_2$ | NERBCs | CC cathode and a glucose | CC anode.

Scheme 1. Assembly diagram of the battery with an O$_2$ | NERBC | CC biocathode construction.
However, once the –OH group on the HAP (002) moved to the side face of Hb, the $d_{Fe-O}$ distance was shortened to 1.76 Å. Moreover, Bader charge analysis showed that, before the attachment of the –OH group on Hb (left) there is 0.08e charge transfer between the Fe and O atoms. After the adsorption of –OH on Hb, the corresponding charge transfer between the Fe and O atoms was increased to 0.12e. This observation further confirmed that the enhanced interaction between Fe and the adsorbed $O_2$ molecule resulted from –OH adsorption on Hb. It is believed that the enhanced interaction between Fe in Hb and $O_2$ upon –OH adsorption weakens the O–O bond, and thus facilitates $O_2$ reduction. In summary, NERBCs were successfully prepared by an in situ synthesis method within the RBCs. This method is based on a mild two-step ion permeation process, in which Hb is combined with Ca$^{2+}$ followed by a phosphate buffer solution (pH 9) containing $PO_4^{3-}$ and OH$^-$, whereupon HAP nano-dots are formed. The formed NERBCs are environmentally friendly, and the product shows excellent biocompatibility with no apparent change in the morphology and permeability relative to the original native RBCs. Their $O_2$ adsorption ability was greatly enhanced, showing superior stability and ORR electrocatalytic ability with an onset potential of 0.47 V (vs. RHE) and a maximum current density of 2.6 mA cm$^{-2}$. This study also reveals the advantages of NERBCs as an ORR electrocatalyst with good selectivity and adhesion to the cathode. All the above unique properties could lead to the application of durable, miniature, separator-free BFCs with good biocompatibility. From the experimental results and computational calculations, the superior performance of NERBCs as compared to natural RBCs is due to the controlled formation of HAP through biomimeralization. The interaction between the –OH groups in HAP and the Hb in RBCs aids the combination of Fe$^{2+}$ and $O_2$, which consequently enhances the adsorption of $O_2$. The BFCs based on an $O_2$ cathode, a glucose anode, NERBCs as the catalyst, and simulated human blood as the electrode exhibited extraordinary performance with an open-circuit voltage of 0.636 V and a maximum energy density of 21.11 μW cm$^{-2}$. This research provides a promising strategy for the development of novel biocompatible electocatalysts and offers a new direction for fabricating functional nanoengineered cells.

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**Conflict of interest**

The authors declare no conflict of interest.

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