Nanobiomimetic Memristor/Memcapacitor Devices Used for Direct and Reagent-less Detection of Sub pM Acetyl Coenzyme A in Milks

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ABSTRACT

Milk quality is key to the wellness of human babies, and detecting essential benefits and critical deficiencies in different types of milk is a crucial element to assuring good brain development. Acetyl co-enzyme A (AcCoA) is a leading substrate in a large variety of enzyme-catalyzed reactions. Unbalanced regulation of AcCoA leads to many diseases, such as diabetes, cancer, coronary disease, schizophrenia, sudden infant death syndrome and Alzheimer's. We developed a biomimetic memristor/memcapacitor sensor for direct measure sub pM concentrations of AcCoA under the conditions of free from antibody and labeling tracers, and is reagent-less. The signal changes of AcCoA over a wide concentrations range between 2 pM to 0.3μM were measured by the chronoamperometry (CA) method; it has a linear range from 2 pM to 0.4 nM. The value of Detection of Limits (DOL) is $1.2x10^{-12}M/cm^2$. AcCoA spiked milk specimens were measured with a recovery value of 103%, and the method produced an error of less than 2% (n=12) by using milk samples. We studied the energy impact of human milk and USDA certified organic milk for infants upon the neuronal synapse action / resting potential pulses with and without AcCoA at slow-wave-sleeping (SWS) and 250 Hz, respectively. The results indicate that human milk has a 5-fold higher energy outcome for infants than organic milk at SWS for benefiting brain development, and human milk is orders of magnitudes sensitive for communicating with the sensor when AcCoA occurred than in organic milk when a double step chronopotentiometry (DSCPO) method was used.

Keywords: Nanobiomimetic Neural Memcapacitor; Voltage Sensor and Amperometric Sensor; Reagent-less; Memristor; AcCoA.

INTRODUCTION

Acetyl co-enzyme A (AcCoA) is a leading substrate in a large variety of enzyme-catalyzed reactions, such as for choline acetyltransferase (CHAT) and acetylcholinesterase (ACHE) [1-5]. Szutowicz's group emphasized that AcCoA is the key factor for the survival or death of cholinergic neurons in course of neurodegenerative diseases [5]. Ivan Gout's group emphasized that the level of AcCoA is crucial to early embryonic development [6]. AcCoA is a thioester derived from catabolism of all major carbon fuels. AcCoA may play a role in the energy production, metabolism, memory, cell proliferation and early childhood development, and it is central to biological acetylation reactions. AcCoA deficiency leads to many diseases, such as diabetes, cancer, coronary disease, autism, Alzheimer's, and sudden infant death syndrome. Abnormality of CHAT activity may lead to these diseases because CHAT represents the most specific cholinergic marker in the CNS [7-8], and the spatial temporal manifestation of CHAT has been examined at both the protein and mRNA levels in different tissues of various species [8].

Furthermore, reports revealed that the virus replications of West Nile virus (WNV), the neurotropic flavivirus that is transmitted by mosquito bites causing meningitis and encephalitis in humans [9], involved the carboxylation of AcCoA to malonyl CoA through AcCoA carboxylase [9]. Therefore, sensitive quantitation of the CHAT activity, in terms of monitoring the changes of substrate AcCoA in biological specimens, is on demand for monitoring and diagnosing various diseases.

Challenges exist for providing a non-enzymatic label-free, reagent-less detection device for the direct detection of AcCoA with rapid detection time, free specimen preparation, and pM high sensitivity are paramount in order to avoid time-consuming assays and protein interferences. Many native enzymatic methods reported to detect AcCoA have the concentration range between mM to μM, such as the CoA cycling method [3], the carbon radioactive tracer labeling method [10-11], and the gas chromatography-mass spectrometry method [12]. The HPLC antibody method can reach to 0.1μM level of AcCoA [6]. In view of the drawbacks of these methods, none of these methods can provide adequate sensitivity in pM level and the short testing time needed for testing AcCoA inside of the mitochondria cell when newborns consume human milk compared with that of cow milk in order to monitor the quality of the milk for babies.

It is well accepted that breast-feeding offers more benefits for human babies' growth in nutritional content and immune defense support over that of cow milk consumption [13-15] and it is a strong recommendation published by the World Health Organization [15]. However, to actively pursue real-time monitoring of breastfeeding and obtain the

preliminary data using an innovative device is not practically feasible now. The goal of this project is to develop a nanostructured memcapacitor/memristor sensor for antibody-free, reagent-less direct measure pM AcCoA at different frequencies to assess the energy outcome comparing human milk with cow milk without protein interference and in a real-time and sensitive manner. The memcapacitor/memristor device will represent, in concept, a human infant single brain neuron's ability to "feel" or sense the energy gain or loss that is due to the presence of AcCoA signaling with the biomimetic CHAT of the sensor membrane in a biological specimen. This project is based on our prior experience in memristor/memcapacitor to mimic hippocampus-neocortex neuronal network circuitry [16-20].

EXPERIMENTAL

Fabrication of the Nanostructure Self-Assembling Membrane (SAM) Gold Memristor Chips

The nanostructured biomimetic SAM was freshly prepared according to the published procedures based on cross linked conductive polymers of triacetyl-ß-cyclodextrin (TCD), polyethylene glycol diglycidyl ether (PEG), poly(4-vinylpyridine) (PVP) and ß-CD copolymer with appropriate amount of propositions on a 16 channel gold chip [21-22]. The chemicals were purchased from Sigma and purification procedures were conducted before use. A mixture of o-nitrophenyl acetate (o-NPA) in a molar ratio 1000:1 to the TCD mixture was incubated for 2 hours at 35°C, and then the mixture was injected onto the gold surface and incubated for 48 hours at 35°C, and after that, clean procedures for completion of the SAM fabrication were followed [21-22].

Characterization of the Membrane

The morphology of the AU/SAM was characterized using an Atomic Force Microscope (AFM) (model Multimode 8 ScanAsyst, Bruker, PA). Data was collected in PeakForce Tapping Mode. Probes used were ScanAsyst-air probes (Bruker, PA). The silicon tips on silicon nitride cantilevers have 2-5 nm radius. The nominal spring constant 0.4N/m was used. Fig. 1 shows the 3D AFM image before the o-NPA was embedded on gold. An energy storage device made with GC/TCD/PEG/PVP/copolymer embedded with o-NPA reported enhanced device energy and power density in 1 M methanol compared without o-NPA [22].

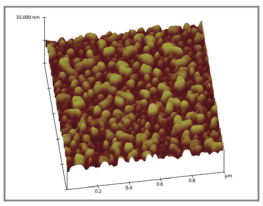

Fig. 1 shows the 3D AFM image of a gold/SAM before adding o-NPA.

Frequency Affects on Memristor/Memcapacitor's Performance

Evaluations of frequency affect on memristors' performance were conducted by Cyclic Voltammetric method (CV) in pH 7.0 saline solution at room temperature. The scan rate is from 1 Hz to 1KHz in PBS pH 7.0 buffer. The data were used for comparing with a fresh human milk in the presence of 60 pM AcCoA and 2 mM o-NPA covering the same range of frequencies against two controls, explained at above section.

Quantitation of AcCoA

The chronoamperometric (CA) method was used for quantitation of AcCoA. The data were acquired at room temperature under two-step fixed potentials in 6 concentration levels covering AcCoA final concentrations ranging from $2.0x10^{-12}$M to $4.0x10^{-10}$M, with triplicates in pH 7.0 PBS in the presence of 2 mM o-NPA against 2 controls, one with 2 mM o-NPA, and another control without o-NPA. Accuracy was accessed by organic milk specimen samples with 60pM spiked AcCoA, run triplicates; obtain the signal and then using the sample signal divided by the data obtained from the calibration curve to obtain the percentage of recovery. An electrochemical work station was used (Epsilon, BASi, IN) with a software package from BASi. Origin Pro 2016 (Origin Lab Corp., MA) was used for all statistic data analysis and figure plotting.

Assessing Energy Outcomes

The Double Step Chronopotentiometry (DSCPO) method was used for assessing energy outcomes of slow-wave-sleeping (SWS) at 0.25Hz compared with fast gamma oscillation frequency at 250Hz under the influence of AcCoA by using human milk vs. USDA certified organic cow milk for infants. It was conducted against controls. Human milk was collected from a normal subject who breastfeeds a 1 month-old newborn (Leebio Corp.,). Each specimen run included triplicates at each of the two frequencies for with or without AcCoA at ±10 nA, respectively.

RESULTS AND DISCUSSIONS

Advantage of AcCoA's Rate Limiting Binding

We thought using the function groups in the SAM membrane to mimic the AcCoA's human choline acetyletransferase (CHAT) binding sites intrinsically to the mitochondria double membrane compartment with the structure needed may be a simplified approach as a neuronal sensor model. The model of the device is to mimic CHAT's function in emphasizing of AcCoA's rate limiting step binding [1-5]. The possible electron-relay was proposed by the pyridine group in PVP, the COO^- group of TCD, the OH group from β-CD copolymer, and the carbonyl group from o-NPA through hydrogen bindings to be able to mimic s540, y552, c563, c550 and h324 of AcCoA binding sits in CHAT. The innovative approach is to first direct detect AcCoA in the mimicking binding sites of CHAT, without choline participates in the direct detection of AcCoA.

Frequency Affects on Memristor/Memcapacitor's Performance

Fig. 3's i-V hysteresis curve was demonstrated by a switch point at the origin (0, 0) at almost all frequencies, except at kHz high frequency in control media PBS only. This perfect hysteresis behavior especially peaks at SWS frequency with sensitive *Direct Electron-relay Transfer* (DET), and the switch point originating at the origin represents a healthy "newborn single neuron" that exists before feeding. Nonlinear frequency influence on current intensity is a characteristics of the memristor reported in literature [17-20, 23-26]. Fig. 4 depicts the high dose intrinsic cellular AcCoA concentration negative effect on the healthy neuron's performance, even when feeding human milk, as evidenced by eliminating the original sensitive DET_{red} peak, which led to three consequences: lowering the peak intensity at SWS frequency; neuron loses its sense of or detection of danger in the presence of toxins; moving the cross-point far from origin at 1 Hz. The data implied that the memrisor sensor provided a mitochondria-like intrinsic double membrane environment that demands a lower pM concentration of AcCoA inside the neuronal cell than in extracellular media when baby is at SWS [5].

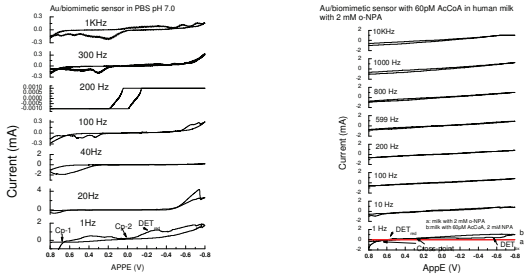

Fig 3 (L) Illustrates the frequency effects from 1 Hz to 1 KHz on hysteresis of the i-V curve of the memristor in PBS. Fig 4 (R) depicts frequency effects with 60 pM AcCoA in human milk with 2 mM o-NPA.

Quantitation of AcCoA

Fig. 5 illustrates CA curve profiles of AcCoA over the linear range of $2.0x10^{-12}$M to $4.0x10^{-10}$M in the presence of 2 mM o-NPA. The role of o-NPA is for enhancing of the hydrophobicity [16, 18-19], and it has no signal interference with AcCoA as shown in the control. The profiles show the signal intensity is in direct proportion to the increase of the AcCoA concentration. Fig. 6 illustrates the calibration curve with a linear regression equation Y= 2.1 + 57X, r=0.994 (n=15), P<0.0001, Sy/x=0.95. The value of Detection of Limits (DOL) is $1.2x10^{-12}$M/cm^2. Because this sensor is only 0.031 cm^2, hence, its DOL is 37 fM in PBS. The measurements can be extended to an exponential nonlinear model from 2 pM to 0.30 µM with y = A1*exp(-x/t1) + A2*exp(-x/t2) + y0, y0=1225.5, A1= -916, A2=-372, t1=0.964, t2= 64.5, Chi^2/DoF= 17255.7, r= 0.98, n=27, 9 levels. Curve fit was not shown. The recovery value using milk at 60pM AcCoA is 103±2%. The imprecision of milk samples in 60 pM AcCOA is 1.75% (n=12).

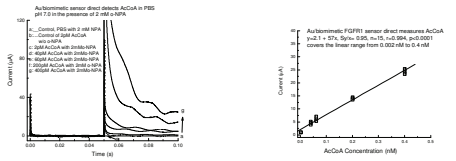

Fig. 5 (L) illustrates CA curve profiles and Fig. 6 (R) shows the calibration plot of current vs. AcCoA concentrations.

Assessing the Neuronal Network Sensory

The DET and Cross-point. We studied a "neuron" memristor's performance in i-V curves using fresh human milk with or without 60 pM AcCoA as shown in Fig. 4. Fig. 7 and Fig. 8 depict the sensor responded to human milk and organic cow milk differently. For example, at 1 Hz, the sensor has more DET peak and cross-point with human milk compared to cow milk, which has none, regardless of whether the samples are tested with or without AcCoA. These figures revealed human milk provides the single neuron a critical sensory function at the memory consolidation stage of brain development, and safe guards the reversible membrane potential in place, and ensures the normal function of direct electron-relay. The possible source or cause may be the contribution of the good bacteria as compared to the cow milk, which has none [27], because pasteurization of cow milk destroys both good and bad bacteria [27]. Another source which may contribute to the brain development may be the unique proteins such as A2 β-casein, which is plentiful in human milk, and lacking in cow milk [28]. Fig. 5 shows there are no DET peak and no cross-point occurrence at 1 Hz with organic milk, and that indicates the cow milk offers a disadvantage for infant development compared with human milk. In contrast, at 100Hz, the cross-points and DET peaks showed up in the i-V curve when spiked with AcCoA, and that may be the key reason causing energy drainage as evidenced in following sections. However, a dosage of AcCoA at 60 pM does do damage to the neuron even when using human milk, as evidenced by the moving away of the cross-point from the origin, and the energy was reduced as shown in the following section.

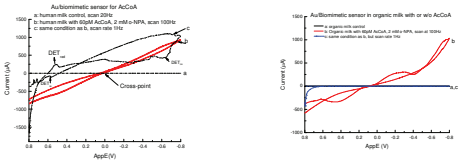

Fig. 7 (L) Depicts the CV profiles in human milk with and w/o AcCoA. Fig. 8 (R) illustrates the CV curves with organic milk with and w/o AcCoA.

Assessing Energy Outcomes

Assessing the energy outcomes were conducted by comparing human milk and the USDA certified organic cow milk for infants, both with and without 60 pM AcCoA, at 0.25 Hz and 250 Hz, respectively, using the DSCPO method. Fig. 9A depicts the 60 pM AcCoA reduced the synapse voltage discharge by 94% at 0.25 Hz in human milk compared without AcCoA. AcCoA reduced more energy outcome at SWS as compared at 250Hz, as shown in Fig. 9B. Also shown, the good bacteria in human milk boosted the net

energy of five-fold, 1.04 nWHr/cm^2 compared 0.19 nWhr/cm^2 of organic milk without AcCoA at 0.25Hz; and 25.3 pWHr/cm^2 with human milk compared with 37 pWHr/cm^2 of organic milk in the presence of 60 pM AcCoA at 0.25Hz as shown in Fig 10. From these results, human milk offers great benefits than organic cow milk for the development of neuronal cells at all frequencies studied regardless of whether it contains AcCoA. The biphasic synapse curves with the highest intensity at SWS over other frequencies are the characteristics of the normal human brain function, and it originating energy for memory consolidation demonstrated using the nanobiomimetic memristor/memcapacitor device using human milk. Using the same device has a destroyed biphasic synapse pattern with so low energy outcome using organic milk as shown in Fig. 10 at 0.25 and 250Hz, with and without AcCoA, indicates there is an urgent need to enrich the probiotic in the cow milk for children. All evidences presented here clearly and convincingly shows that human milk increases the energy available to output to the neuronal cell for further brain developing, and which supports a strong recommendation by the authors to women to seriously consider the advantages of breast-feeding to infant brain development and health.

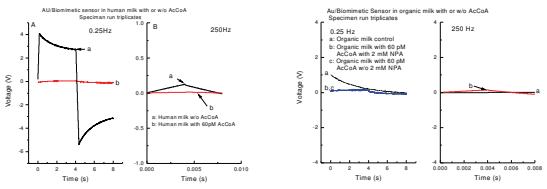

Fig. 9 (L) depicts the sensor's detections of AcCoA in human milk with or without AcCoA at 0.25 Hz and 250Hz, respectively. Fig. 10 (R) depicts the sensor detecting AcCoA in certified organic milk at 0.25 Hz and 250Hz, respectively.

CONCLUSION

We have demonstrated the advantage of binding AcCoA to a nanostructure biomimetic CHAT membrane enabled the sensor to direct, fast, sensitive and reliable detecting AcCoA without the presence of choline, without antibody, and no tracer, no probes and is reagent-less. We demonstrated this sensor is able to detect intrinsic pM level of AcCoA in the biomimetic mitochondria cell of a single neuron, so that the energy outcomes with and without AcCoA can be monitored in real-time covered with wide-band synapse frequencies from SWS to fast gamma ripples for monitoring milk quality and deficiencies for improving of infants health.

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