闭合式无线纳米孔电极检测单个外泌体

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摘要 单个体电化学通过在限域电化学测量界面上检测单个纳米粒子、单个脂质体、单个细胞等的电化学特性, 实现对单个体各向异性的研究. 这种高度灵敏的电化学方法能够快速测量并区分纳米级单个体的大小和表面电荷等. 目前, 已在单颗粒催化、环境监测和细胞分析等领域得到应用. 本研究通过使用尺寸可控的石英纳米孔作为模板, 将贵金属金沉积在石英纳米孔尖端, 以形成用于单个体测量的纳米级电化学限域界面, 从而制备具有高灵敏度的闭合式无线纳米孔电极. 利用该电极构建了单个体电化学测量体系, 实现了单个外泌体的检测, 获得了信噪比高达25.1的单个外泌体碰撞信号, 电化学测量平均电流幅值为15.1 pA, 单个信号持续时间为0.4 ms, 从而实现了单个外泌体与纳米电极间动态相互作用的实时测量.

关键词 无线纳米孔电极, 纳米孔, 纳米电极, 电化学空间限域效应, 外泌体, 单个体电化学

单个体电化学通过电化学方法研究单个纳米粒子^[1-5]、单个脂质体乃至单个细胞的电化学特性. 这一高度灵敏的电化学测量方法, 能够快速测量和直接区分纳米粒子尺寸和表面电荷等特性, 已经实现了单个纳米粒子在催化^[6]、细胞分析^[7]、环境监测^[8]等方面的应用. 目前, 单个体电化学的检测应用策略主要分为3种: 基于碰撞原理的微纳米电极技术^[9-11]、基于电阻-脉冲原理的纳米通道技术^[12-15], 以及基于微纳电化学测量和其他方法联用的多通道测量技术^[16-20]. 1995年, Bard^[21]首次报道了通过使用纳米尺寸电极实现单分子电化学分析. 随后, 微/纳米级电极被应用于单个纳米颗粒的高灵敏测量^[22,23].

前期利用石英纳米孔为模板, 在纳米孔中沉积贵金属金用作双极电极, 从而在纳米孔界面形成纳米尺

度的电化学限域测量界面, 使两极在不直接与驱动电极连接的情况下获得电化学测量信息^[24]. 这种在纳米孔内部构建与驱动电极无直接连接的氧化还原界面的闭合式无线纳米孔电极(CWNE), 其测量界面具有均一的尺寸和形貌, 具有电流分辨率高、时间分辨率高的特点. 基于此, 本研究通过构建闭合式无线纳米孔电极, 获得高时间分辨率和高电流分辨率的纳米限域测量界面, 并进一步将基于闭合式无线纳米孔电极的电容反馈(capacitive feedback response)单个体测量机制^[24-26]应用于单个外泌体测量中. 外泌体^[27,28]是一种可以由大多数细胞分泌的富含复杂RNA和蛋白质的膜囊泡, 主要来源于细胞内溶酶体内陷形成的多囊泡体, 其大小为40~100 nm, 是细胞通讯的重要介质. 在疾病机理、药物开发、基因诊疗^[29]等方面有广阔的应用潜

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力。因此,本研究以外泌体作为研究对象,在电场极化下,通过单个外泌体和闭合式无线纳米孔电极的限域测量界面相互作用引起的反馈电容增加,从而实现单个外泌体的实时测量,进一步推动单个体电化学在生命分析中的应用。

1 实验

(i) 试剂与仪器。实验中使用的丙酮、乙醇、氯化钾、氯金酸、硼氢化钠、硝酸钾均购自Sigma-Aldrich(St. Louis, MO, 美国),试剂纯度均为分析纯;石英毛细管购自Sutter Instrument(Novato, CA, 美国)。实验用水均为超纯水($18.2 \text{ M}\Omega \text{ cm}^{-1}$, 25°C)。

实验中产生的电流信号使用Axon 200B膜片钳放大器 and DigiData 1440A A/D转换器(Molecular Devices, Forest City, CA, 美国)进行放大和采集。电流信号经由5 kHz低通滤波、100 kHz采样频率采集后通过Clampex 10.4程序获得(Molecular Devices, Forest City, CA, 美国)。数据处理分析使用OriginLab 8.0(OriginLab Corporation, Northampton, MA, 美国)软件。P-2000 CO_2 激光拉制仪购自Sutter Instrument(Novato, CA, 美国)。

(ii) 石英纳米孔的制备。清洗石英毛细管:用镊子取出用于制备石英纳米孔所需的石英毛细管(外径1.0 mm, 内径0.5 mm, 长度100 mm),放置在15 mL离心管中,加入没过石英毛细管高度的丙酮,拧紧离心管盖子,超声清洁20 min。超声结束后,移去丙酮,重新分别加入乙醇和超纯水,超声清洁20 min。移去多余的超纯水,用干燥洁净的氮气将石英毛细管吹干,并置入干燥洁净的15 mL离心管中,在恒温干燥箱中放置保存。

P-2000 CO_2 激光拉制仪制备石英纳米孔:将干燥洁净的石英毛细管装载入拉制仪中,设置参数(P1 [HEAT=650; FIL=3; VEL=35; DEL=145; PUL=75]; P2 [HEAT=750; FIL=2; VEL=15; DEL=128; PUL=200])拉制石英毛细管,可以得到两根镜像对称的石英纳米孔。将制备的石英纳米孔置于干燥洁净的培养皿中保存。

(iii) 石英纳米孔和外泌体的形貌表征。表征同一拉制参数制备得到的石英纳米孔的孔径。基于同一根石英毛细管拉制成两根镜像对称的石英纳米孔,一根用于电流-电压(I - V)曲线表征^[30],另一根用于扫描电子显微镜(SEM)测量孔径大小。

I - V 曲线表征:用移液枪抽取10 μL 的1 mol L^{-1} 氯化钾(KCl)溶液注入石英纳米孔内部,将石英纳米孔以尖端朝下的方式固定在离心管中,离心5 min(转速3500

r min^{-1} , 离心力 $1878\times g$),移除气泡,使纳米孔尖端充满溶液。取出石英纳米孔,用硅胶将石英纳米孔固定在洁净玻璃片上并划分内侧和外侧。将两根银/氯化银(Ag/AgCl)电极分别置入石英纳米孔的内侧和外侧,外侧滴加1 mol L^{-1} KCl溶液,设定电压扫描范围为 $-1\sim 1 \text{ V}$,施加电压间隔为100 mV,每次施加电压持续时间10 s,可以得到该石英纳米孔的 I - V 曲线。

SEM表征:使用EVO18扫描电子显微镜(Carl Zeiss AG, Oberkochen, 德国),电压为5 kV来表征石英纳米孔。以正对石英纳米孔开口处方向直接观察测量石英纳米孔的孔径。

实验中的外泌体由中国人民解放军第二军医大学第三附属医院提供。外泌体的形貌大小使用JEM-2100透射电子显微镜(TEM, JEOL Ltd., Tokyo, 日本)表征,加速电压为100 kV。

(iv) 闭合式无线纳米孔电极的制备。用移液枪抽取10 μL 的1 wt%(重量百分比)氯金酸(HAuCl_4)溶液,注入石英纳米孔内部。将石英纳米孔以尖端朝下的方式固定在离心管中,离心5 min(转速3500 r min^{-1} , 离心力 $1878\times g$),移除气泡,使纳米孔尖端充满溶液。取出石英纳米孔,用硅胶将石英纳米孔固定在洁净玻璃片上并划分内侧和外侧。将两根Ag/AgCl电极分别置入石英纳米孔的内侧和外侧,外侧滴加无水乙醇,构建液-液界面。两根电极分别连接在Axopatch 200B电流放大器的前置放大器接口上,在两电极间施加100 mV电压,在Clampex 10.4软件中记录电流变化。实验期间,使用特制的金属屏蔽箱屏蔽外界电磁噪音干扰。

将无水乙醇移除,在外侧加入10 mmol L^{-1} 硼氢化钠(NaBH_4)的乙醇溶液。 AuCl_4^- 可被 BH_4^- 还原为金,从而堵塞纳米管孔口。乙醇可以减缓 AuCl_4^- 和 BH_4^- 的反应速率。没有乙醇的存在, NaBH_4 会在水溶液中以剧烈的反应速率还原为 H_2 ,这将会导致缺陷结构的形成。一旦在尖端产生金, AuCl_4^- 和 BH_4^- 之间的化学反应被阻隔。此时,所产生的金尖可能是不规则的。随后,双极电化学在电场作用下促进了在导体金尖的两端发生的两个电子转移过程。触发双极电化学反应的唯一条件是在导电体两端有足够的电位,该电位需要大于一对氧化还原反应所需的电位。施加300 mV的电位,可以进一步在纳米孔尖端增长金尖。利用电化学延长了金纳米尖端,极大地增加了尖端两端的极化差异。此时闭合式无线纳米孔电极制备完成。同时,电流曲线会呈现先下降后升高,最后趋于0 pA的变化趋势(图S1)。

(v) 单个外泌体在CWNE上的测量. 取10 mL 10 mmol L⁻¹的硝酸钾(KNO₃)溶液于15 mL离心管中, 再取10 μL外泌体混合液(2 μg mL⁻¹, 10 mmol L⁻¹的PBS缓冲液), 加入离心管中, 稀释外泌体1000倍. 封闭式无线纳米孔电极制备完成后, 移去溶液池的硼氢化钠/乙醇溶液, 用超纯水沿着尾端到尖端的方向连续冲洗CWNE 20次. 将配置好的外泌体溶液加入到冲洗过后的溶液池中, 用两根Ag/AgCl电极分别连接CWNE的内侧和溶液池. Ag/AgCl电极再与放大器前置探头连接, 经过电流放大器获得单个外泌体与电极发生相互作用的电流反馈.

2 结果与讨论

2.1 石英纳米孔的*I-V*曲线和SEM形貌

图1(c)是采用同种参数控制的石英纳米孔进行*I-V*曲线表征结果. 利用同种参数获取3组石英纳米孔, 随机取每组中一根石英纳米孔进行*I-V*曲线表征, 计算得到曲线斜率, 分别为0.078, 0.083和0.074 nA mV⁻¹. 通过下列公式^[31]计算孔径:

$$R = \frac{\rho l}{\pi r_s r_t}, \quad (1)$$

其中, 纳米孔的电阻为 R , 电解质溶液的电阻率为 ρ , 石英管的长度为 l , 石英管的半径为 r_s , 尖端的半径为 r_t . 计算可知*I-V*曲线表征得到的孔径为50.8, 54.0以及48.2 nm. 再取另一根同组控制的石英纳米孔, 进行SEM表征, 结果显示分别为52.2, 58.7和47.6 nm(图S2). 从结果可以得到, *I-V*表征得到的石英纳米孔孔径与SEM测量得到的孔径相符合.

2.2 CWNE上外泌体碰撞信号的电流响应

为了研究CWNE的稳定性, 在-500~500 mV的外加电压范围内, 每隔100 mV记录基线电流变化情况. 此时, CWNE外部溶液是10 mmol L⁻¹的KNO₃溶液. 从图2(a)中可见, 在不同电压下离子流约为0 pA, 变化幅度不大, 说明金在石英纳米孔尖端沉积较好, 离子流影响小, 形貌稳定. 电流基线噪音为(5.6±2) pA, 均方根(RMS)值为(0.6 ± 0.1) pA(图S3), 说明构建了稳定的低噪音界面.

利用SEM表征外泌体的形貌, 由图3(a)可以看出, 外泌体的直径大小约为30 nm, 各个外泌体之间形状均一, 大小相似. 图1(a), (b)所示是在CWNE上发生单个外

图1 (网络版彩色)可重复制备的CWNE用于获取单个外泌体碰撞电流信号. (a) 单个外泌体与CWNE碰撞示意图; (b) 单个外泌体与CWNE碰撞产生的单个电流信号; (c) 石英纳米孔的*I-V*曲线表征; (d) 石英纳米孔SEM图

Figure 1 (Color online) The reproducible CWNE for single exosome sensing via collision current signal. (a) Schematic diagram for a single exosome collision towards a CWNE. (b) A current signal generated by the collision of a single exosomes with CWNE. (c) *I-V* characterization of quartz nanopores. (d) A SEM image of a quartz nanopore

图2 (网络版彩色)CWNE的制备表征及外泌体测量原始离子流电信号. (a) 单个CWNE的离子流电信号表征. 施加电压扫描范围: -500~+500 mV; (b) 单个CWNE测量外泌体碰撞的原始离子流电信号响应. 施加电压: 500 mV; (c) 单个外泌体碰撞所产生的个体原始离子流电信号i-vii

Figure 2 (Color online) The electrochemical characterization of a CWNE and its application on single exosomes collision. (a) The raw current traces for characterizing a well-prepared CWNE under applied potential ranging from -500 to +500 mV. (b) The raw current traces of exosomes collision at a CWNE under applied potential of 500 mV. (c) Ion current signals i-vii of individual exosome collisions

图 3 (网络版彩色)外泌体形貌表征和利用CWNE的碰撞信号统计分析。(a) 外泌体SEM表征; (b) CWNE与外泌体碰撞信号的电流-基线差值统计直方图; (c) CWNE与外泌体碰撞信号的电量变化值统计直方图; (d) CWNE与外泌体碰撞信号的持续时间统计直方图

Figure 3 (Color online) The characterization of exosomes and statistical analysis of collision signals using CWNE. (a) SEM characterization of exosomes. (b) Current histogram of exosome collision signals. (c) Histogram of integrated charge difference for exosome collision signals. (d) Duration histogram for exosome collision signals

泌体碰撞产生信号电流的示意图。CWNE两侧施加电压后,在纳米孔尖端的沉积金上产生极化作用。单个外泌体与CWNE相互作用会改变测量界面上的电荷分布。基于前期对CWNE提出的电容效应检测机制^[24],外泌体表面带负电荷^[27],在CWNE表面碰撞时通过调制充放电过程,从而获得单个反馈电流,实现单个电化学测量。如图1(b)所示,单个外泌体信号响应的持续时间 t 约为1 ms,电流-基线差值 i 约为15 pA,即对于单个事件的检测具有高电流分辨率和高时间分辨率。

如图S3所示,相比500 mV情况下,施加300 mV时的单个外泌体碰撞频率低。因此,在500 mV电压下研究了单个外泌体与CWNE的相互作用。图2(b)是在500 mV条件下记录电流-时间曲线,其具有单个信号频率高、信噪比高的特点。这一结果说明制备的CWNE可用于单个外泌体的非标记、高灵敏检测。

图2(c)显示在500 mV电压条件下,不同的单个电流事件的形状差异。信号i~vii是外泌体与CWNE发生瞬时碰撞而产生的单个电流事件。由于单个外泌体在个体尺寸、形貌、表面电荷分布等方面的差异性,造成了单个外泌体与CWNE相互作用时碰撞部分的电荷分布及电荷密度差异。因此,每个单独信号的电流-基线差值 i 以及持续时间 t 的差异,主要是来自于单个外泌体与CWNE相互作用时的个体性差异。

统计电流响应信号,分别提取每个单独信号的电流-基线差值(i)、信号持续时间(t),并计算每个电流响应信号在持续时间内的积分电流值(即单个信号的电量变化值)。图3(b)~(d)分别对所有信号的电流-基线差值(i)、电量变化值、持续时间(t)进行直方图统计并进行拟合,结果显示,三者均符合高斯分布。其中,电流-基线差值 i 拟合峰值为15.1 pA,计算得到信噪比为25.1(补充材料),说明CWNE检测外泌体的电流响应信号具有高信噪比。电量变化值的高斯拟合峰值为2.7 fC,说明带负电荷的外泌体在与CWNE作用过程中改变了测量界面上的电荷分布,电流信号反映了电荷的传递过程。持续时间(t)的高斯拟合峰值为0.42 ms,说明CWNE可以检测事件的毫秒级时间变化。另外,SEM表征和CWNE上电量变化情况,均说明外泌体的形状和带电量的分布都较为均一。

3 结论

本研究通过构建纳米尺度电化学限域测量界面,测量了直径大小为30 nm的外泌体与限域电化学测量界面间的相互作用,获得了皮安级电流分辨以及毫秒级时间分辨。单个外泌体信号的电流统计幅值为15.1 pA,持续时间统计值为0.42 ms,信噪比为25.1,实现了对单个外泌体在利用闭合式无线纳米孔电极在纳米尺度的检测。这一结果将闭合式无线纳米孔电极的检测范围从单个颗粒拓展到了单个外泌体,可推动其在生命分析领域的发展。进一步,可发展特异性修饰的CWNE,使电极具备对外泌体上蛋白的选择性,从而具备在复杂的混合样品中对外泌体特异性检测的能力。

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图S1 CWNE制备过程原始电流曲线

图S2 SEM表征同一参数拉制的石英纳米孔径

图S3 300 mV电压下CWNE检测单个外泌体的原始电信号记录曲线

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Summary for “闭合式无线纳米孔电极检测单个外泌体”

Detection of single exosomes with a closed wireless nanopore electrode

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Single entity electrochemistry has been used to detect the intrinsic properties of individual nanoparticles, liposomes and cells at the electrochemical measurement interface. This highly sensitive electrochemical method enables rapid measurement and direct discrimination between nanoparticle size and surface charge. It has been used in the field of single nanoparticle catalysis, environmental monitoring and cell analysis. The sensing interface of nano-/micro-electrode is essential for performing an ultra-sensitive single entity measurement. In this study, a highly sensitive closed-type wireless nanopore electrode (CWNE) was fabricated for single entity electrochemistry. Using a morphologically controllable quartz nanopore as a template, the nanoscale gold metal was deposited as a bipolar nanotip to form a nanoscale electrochemically confined interface for single entity measurements. Here, the nano-scale electrochemical sensing interface was constructed by injecting AuCl_4^- into the quartz nanopore and placing BH_4^- in the outside solution. These two solutions stimulated the AuCl_4^- reduction BH_4^- with at the tip of the quartz nanopore, thereby forming gold nanostructure at the tip of the nanopore. Considering both the tip configuration of the quartz nanopore and the bipolar electrochemical principle, the applied voltage was dropped mostly on the tip confinement of the CWNE, leading to two polarized terminals at the gold nanotip. The further electrochemical extension of the gold nanotip greatly increased the polarization difference at the ends of the tip. The diameter of quartz nanopore was evaluated in the electrolyte solution by I - V curve, while scanning electron microscopy (SEM) characterization was performed to provide a straightforward characterization under vacuum environment. By using this method, electrochemical measurements could be made at the two terminals of the polarized gold nanotip without directly connecting to the electrode. The fabricated CWNE provides a uniform diameter of sensing interface, controllable morphology, high current and time resolution and low current noise.

In this study, we further applied CWNE to detect individual exosomes with biological activity. Exosomes are membrane vesicles containing complex RNA and proteins, which are mainly derived from the formation of multivesicular bodies formed by intracellular lysosomes. Transmission electron microscopy (TEM) characterization of the exosomes showed a uniform diameter of 30 nm. When using a CWNE to detect the exosomes, a solution containing the exosomes was added to the outside solution of a CWNE. Then, the exosomes approached to the CWNE under the electric field force, and finally collided with the positive terminal of CWNE. Due to the capacitive feedback response, the collision of negatively charged exosomes modulated the charge density at the positive terminal of CWNE. Therefore, the electrochemical interaction between the exosomes and the CWNE interface produced a distinguishable ion current signal. The duration and current fluctuation of the collision signals were further analyzed, and the corresponding charge change was calculated. Our results showed that the frequency of the collision signal increased with the applied positive voltage. Therefore, a larger numbers of collision signals could be obtained under a high applied voltage of 500 mV. Each collision event showed the heterogeneous properties of a single exosome, thus every individual collision signal indicated the characteristic duration, current shape and charge. Our statistical results showed that the single collision signal of the exosomes exhibited a current-baseline difference of 15.1 pA with a duration of 0.42 ms, a signal-to-noise ratio of 25.1 and the calculated change in charge of 2.7 fC. Therefore, the single entity collision of exosomes could be achieved at a CWNE in real time with temporal resolution at millisecond level, which opens a possibility for CWNE in the detection of biological entities with high sensitivity. Our studies could broaden the application of CWNE, which can promote the nano-electrochemical measurement in biological samples.

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