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测定纳米银在河口水中对副溶血弧菌抑制效应的高效方法*

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摘要 由于河口水具有复杂的物理和化学性质, 准确、高效地测定纳米银在其中的抑菌效应是个国际性挑战。本研究基于电子微生物生长分析仪建立了一种自动化表型方法。副溶血弧菌(*Vibrio parahaemolyticus*)在河口水中暴露于纳米银之后, 暴露混合液直接被加进预装有 Luria-Bertani 液体培养基的检测管中, 置入电子微生物生长分析仪测定细菌的生长动力学曲线, 根据细菌生长曲线判读纳米银对副溶血弧菌的最小抑菌浓度。采用电子微生物生长分析仪测定了纳米银在 8 个河口水样品中的抑菌效应, 结果表明, 所得最小抑菌浓度值都与采用经典平板计数法和微量肉汤稀释法所得结果吻合良好。较之于经典方法, 所建新方法的优势在于无需去除暴露混合液中诸如悬浮颗粒之类的复杂共存物, 因此操作更简便、劳动强度小, 总周期缩短至少 20 min, 并有效降低了主观和客观操作误差风险, 具有良好的精密度和重现性。此外, 电子微生物生长分析仪法测得的最小抑菌浓度值通常不低于平板计数法和微量肉汤稀释法所得数值, 表明该基于传感器识别结果的方法比基于视觉判别结果的方法具有更高的灵敏度。本研究为准确、高效测定纳米材料在诸如河口水之类复杂介质中的环境毒理效应提供了新手段。

关键词 纳米银; 生态毒性; 河口水; 副溶血弧菌; 电子微生物生长分析仪

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纳米银(Ag NPs)具有独特的抗菌性能, 是世界上最常用的纳米材料之一, 广泛应用于医疗设备、化妆品、纺织品、电子、玩具和家用电器等领域(Desireddy *et al*, 2013; Loo *et al*, 2018; Bruna *et al*, 2021)。Ag NPs 的广泛应用使它们不可避免地会通过废水、大气沉降和其他途径进入河流、湖泊、河口和沿海水域(Zhao *et al*, 2021; Kang *et al*, 2023), 引发了人们对其生态安全性的担忧(Mühling *et al*, 2009; Yi *et al*, 2017; Li *et al*, 2020)。对于河口环境中纳米材料的风险评估而言, 微生物(尤其是细菌)是理想的指示物(Hegde *et al*,

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2016; Zhang *et al*, 2021)。因此，准确测定纳米材料对细菌的抑制效应是进行环境风险评估活动的必要前提之一(Zhang *et al*, 2021)。

Kirby-Bauer 纸片扩散法、显微镜法、平板计数法、生物量测定法、微量肉汤稀释法(BMD)、电化学传感器、表面增强拉曼光谱法和浊度法(OD)等基于微生物生长分析的表型方法已经被广泛应用于测定纳米材料对细菌的抑制效应(Westmeier *et al*, 2018)。基于遗传物质分析的基因型方法(如 PCR、qPCR、RT-PCR 和高通量测序)由于可以节约细菌培养所需时间，近年来也发展迅速(Doiron *et al*, 2012; Metch *et al*, 2018; Xu *et al*, 2019)。这些表型和基因型方法在测定实验室单纯介质(如培养基)中纳米材料对微生物的影响时具有很好的性能(Westmeier *et al*, 2018; Du *et al*, 2019; Huq, 2020)。然而，当它们被用于测定纳米材料在真实的复杂介质(如河口水样)中对微生物的影响时，必需经过繁琐的分离、纯化等前处理步骤(Niu *et al*, 2021)，不仅效率低，而且主客观误差也在所难免(Westmeier *et al*, 2018; Zhang *et al*, 2023b)。目前，准确、高效地测定实际复杂样品介质中纳米材料抑菌效应的分析方法依然是业界的急迫需求。

近年来，我们团队研制出了一种基于电容耦合非接触电导(C^4)原理的多通道传感器，为在线监测微生物生长过程提供了可能(Zhang *et al*, 2018、2020、2021)。C^4 检测是一种独特的电导率分析方法，工作时电极不与被测试介质直接接触(Chantipmanee *et al*, 2020; Hauser *et al*, 2020; Zhang *et al*, 2020; Tuma, 2022)，输出的信号值正比于介质导电离子浓度及介质中载流子的迁移率。它不仅具有经典接触式电化学分析技术的优点——如仪器简单、成本低廉、响应速度快、不受浊度影响、易于小型化等，而且不存在电极的极化、钝化和污染风险(Zhang *et al*, 2023a)。我们基于该多通道传感器，研制出了 32 通道电子微生物生长分析仪(EMGA)。但是，尚未曾采用 EMGA 建立测定河口水中纳米材料抑菌效应的方法。

副溶血弧菌(*Vibrio parahaemolyticus*)广泛存在于河口及近岸环境中(李昊等, 2023)，是一种条件性致病的革兰氏阴性嗜盐菌(Baker-Austin *et al*, 2010; 黄梦诗等, 2019)。另一方面，它们还具有优异的异养硝化–好氧反硝化能力，能够降解碳氢化合物、木质素等一些难降解的化合物(Grimes, 2020)。因此，探索 Ag NPs 对河口水中副溶血弧菌的影响具有重要的科学意义。本研究以副溶血弧菌为模式微生物，通过采用 EMGA 测定 Ag NPs 胁迫下细菌生长动力学曲线，建立一种分析纳米材料在河口水中抑菌效应的高效方法，探讨该方法的可靠性和实用性，并采用经典的 BMD 法和平板计数法进行性能验证。

1　材料与方法

1.1　细菌培养与计数

副溶血弧菌标准菌株(ACTT17802)购自北京百欧博伟生物技术有限公司，采用 Zhang 等(2023b)报道的方法进行培养。Luria-Bertani (LB)培养基干粉购自青岛海博生物技术有限公司，采用超纯水制成实验用液体培养基(含 0.5 g/L NaCl、10.0 g/L 胰蛋白胨和 5.0 g/L 酵母提取物)。副溶血弧菌在 LB 液体培养基中接种，在 Herocell C1S 型培养箱(上海润度生物科技有限公司, 上海)中于 36 ℃下 150 r/min 轻摇预培养过夜。然后选择活性菌株，转移到新的液体培养基中。第 2 次培养达到指数期后(约 14 h)，取出一部分用标准平板计数法测定细菌浓度(单位 CFU/mL) (Zhang *et al*, 2018)。其余培养液以三平行进行梯度稀释备用。除特殊说明之外，本研究中使用的所有化学试剂均为分析纯。

1.2　Ag NPs 的制备与表征

采用 Bastús 等(2014)报道的湿化学法制备 Ag NPs。50 mL 柠檬酸钠(5 mmol/L)和单宁酸(0.25 mmol/L)的水溶液在三颈圆底烧瓶中油浴，剧烈搅拌下回流 15 min。然后在溶液中注入 0.5 mL 25 mmol/L 的 $AgNO_3$。关闭加热器，让反应持续 10 min。冷却后，取出生成物。通过 3 次离心除去未反应物以纯化产物，最终获得 0.80 g/L 的 Ag NPs 纯水分散液。产物采用透射电镜(TEM, 200 KeV, FEI Talos F200S G2, 美国)、紫外-可见分光光度计(Lambda 900, PerkinElmer, 美国)和粒度仪(Malvern, 英国)进行形貌与性质表征。

1.3　河口水样的采集、分析与处理

河口水采样点信息如表 1 所示，采集时间为 2022 年 4 月和 12 月，方法参照 Das 等(2012)的报道。简而言之，使用 4 L 柱式聚乙烯采水器在水面下 10~15 cm 处采集，然后装在洁净玻璃瓶中于室温下运输到实验室。为去除自然菌群的干扰，样品到达实验室后在 121 ℃下灭菌 30 min。然后于室温下密闭保存，使用前摇匀。

1.4　EMGA 法测定

如图 1 所示，EMGA 法测定 Ag NPs 在河口水中抑菌效应的操作包括 2 个步骤：1)副溶血弧菌急性暴露于 Ag NPs 之后，暴露混合液加入预装有 LB 液体

表 1 采样点经纬度信息

Tab.1 Longitude and latitude information of sampling sites

采样点 Sampling site	纬度 N Longitude	经度 E Latitude
S1	36°11′00″	120°07′25″
S2	36°10′55″	120°07′34″
S3	36°10′45″	120°07′44″
S4	36°14′37″	120°18′31″
S5	36°09′17″	120°09′17″
S6	36°09′11″	120°21′48″
S7	36°01′59″	120°12′00″
S8	37°00′44″	120°11′37″

培养基的检测管中；2)检测管置入 EMGA 中测定存活细胞的生长动力学曲线，从而示出最小抑菌浓度(minimum inhibitory concentration, MIC)。具体如下：将 10^8 CFU/mL 副溶血弧菌接种到灭菌处理过的河口水样品中，使其终浓度为 10^5 CFU/mL。然后各取 10 mL 分装入 6 个 15 mL 离心管中。分别加入倍比质量的 Ag NPs，使该纳米材料的终浓度分别为 0、1.5、3.0、6.0、12.0、24.0 和 48.0 mg/L。室温下，150 r/min 轻摇离心管 30 min，然后各取 200 μL 暴露作用后的混合液分别注入预装有 1.8 mL LB 液体培养基的检测管(外径 5 mm, NORELL 公司, 美国)中。以灭菌 LB 液体培养基作为阴性对照，同样装入检测管。将每个检测管分别插入 EMGA 的一个工作通道中，开启仪器，设置参数——激励电压 12 V、激励频率 400 MHz、采集周期 1 min 和采集次数 1 200，点击“开始”键，EMGA 即实时呈现所有检测管中细菌生长的动力学(ΔC^4-t)曲线。测定结束后，根据是否出现反正弦型生长曲线直接读出生理盐水中 Ag NPs 对副溶血弧菌的 MIC 值(Theophel *et al*, 2014)。

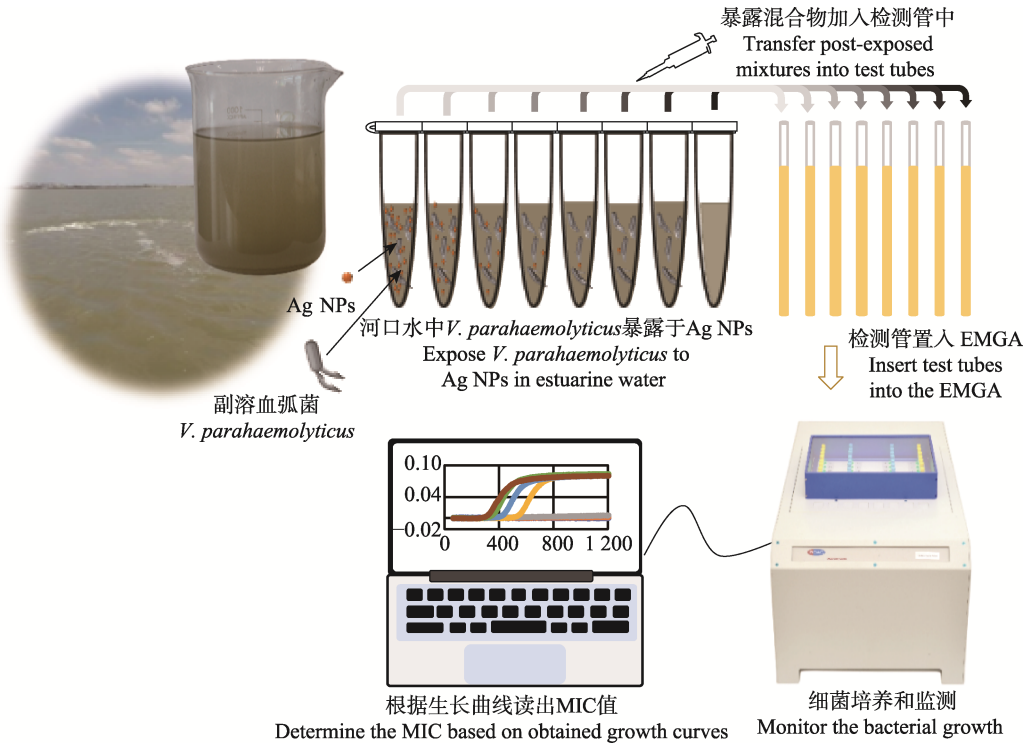

图 1 EMGA 法测定河口水样中 Ag NPs 对副溶血弧菌抑制活性的示意图

Fig.1 Schematic of the working steps of determining MICs of Ag NPs against *V. parahaemolyticus* in estuarine water samples using the EMGA method

1.5 BMD 法测定

根据 CLSI 规定的 BMD 方法(Ceriotti *et al*, 2012)测定河口水中 Ag NPs 对副溶血弧菌的抑制效应。10^5 CFU/mL 副溶血弧菌急性暴露于 Ag NPs 之后，将混合物装入离心管中，采用 Optima XL-90 型离心机(贝克曼库尔特公司, 美国)处理 5 min (3 000 r/min)以去除影响结果判断的悬浮颗粒(Nayak *et al*, 2005)。然后，各取 20 μL 暴露作用后的混合液分别注入预装有 180 μL LB 液体培养基的 96 孔板(康宁生物技术公司, 美国)中(姜晓瑜等, 2021)。用封口膜封住 96 孔板以防蒸发。将 96 孔板放在培养箱中于 36 ℃下培养 20 h，

取出后, 通过肉眼观察是否浑浊判断是否有菌生长增殖, 进而读出 MIC 值。

1.6　平板计数法测定

参照 Feng 等(2020)报道的平板计数法测定河口水中 Ag NPs 对副溶血弧菌的抑制效应。10^5 CFU/mL 副溶血弧菌急性暴露于 Ag NPs 之后, 如无特殊说明, 采用 **1.5** 部分述及的离心法除去混合物中的悬浮颗粒。然后, 采用移液器各取 100 μL 暴露作用后的混合液, 10 倍梯度稀释后, 分别均匀涂在 LB 琼脂平板上。琼脂平板加盖后, 在培养箱中于 36 ℃下静止培养过夜, 取出后, 通过观察是否有菌斑生成判断细菌是否被完全抑制, 进而判断出 MIC 值。

1.7　数据处理与分析

以 BMD 法或平板计数法所测定的 MIC 值为相对标准, 采用基本符合(EA)率值考察 EMGA 法测定 Ag NPs 抑菌活性的有效性。即将 EMGA 法测定的 Ag NPs 对副溶血弧菌的 MIC 值与平板计数法或 BMD 法所测定的 MIC 值比较, 如果正负相差不大于 1 倍, 则视作基本符合(EA); 如果正负相差大于 1 倍而不大于 2 倍, 则视作小偏差(mE); 如果正负相差大于 2 倍, 则视作大偏差(ME)。为了验证 EMGA 法的重现性和精密度, 测定 Ag NPs 在河口水样 S1 中抑菌效应的实验做 9 个平行。采用 Microsoft Excel 2021 处理数据以计算出平行实验结果的平均值和标准差(SD)。

2　结果与讨论

2.1　Ag NPs 的特征

透射电子显微镜(transmission electron microscope, TEM)表征结果显示, 本研究中合成的大部分 Ag NPs 接近球形, 且在水中具有较好的分散性(图 2a)。随机分析 100 个颗粒的粒径(d, nm), 结果显示, 平均值为(37±5) nm(图 2b)。新制备的纳米材料最大光吸收峰出现在 415 nm 左右(图 2c), 表现出 Ag NPs 的特征峰。该结果还表明, 产物具有较好的形貌一致性和纯度。

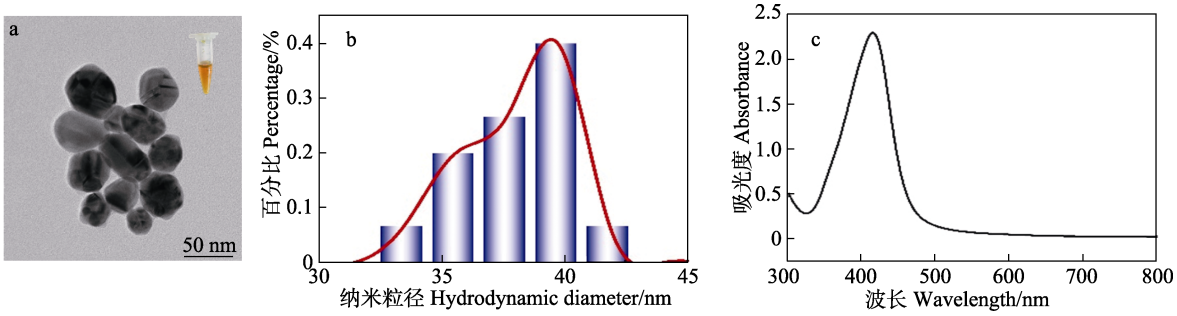

图 2　Ag NPs 的 TEM (右上角插图: 其在水中的分散液照片)(a)、粒度分布图(b)和 UV-vis 光谱图(c)
Fig.2　TEM image (Top right inset showing aqueous dispersion photo) (a), particle-size distributions (b) and UV-vis spectra (c) of Ag NPs

2.2　EMGA 法测定结果

科研界常通过分析纳米材料对微生物生长曲线的影响来推测该被分析物的抑菌效应(Theophel *et al*, 2014)。研究首先以河口水样 S1 作为代表探讨了复杂介质中 Ag NPs 对副溶血弧菌的作用。在河口水样 S1 中, 10^5 CFU/mL 的副溶血弧菌急性暴露于不同浓度的 Ag NPs 之后, 暴露混合液直接转入预装有 LB 液体培养基的检测管中, 采用 EMGA 法测定存活细胞的生长动力学曲线, 结果如图 3a 所示。Ag NPs 浓度为 0 时(阳性对照), 三平行实验皆得到了重现性良好的反正弦型生长曲线, 说明本实验条件下存活细菌很多且正常、稳定地增殖与生长。当河口水中 Ag NPs 浓度逐渐增大时, 副溶血弧菌的生长调整期随之逐渐增加, 直到出现直线型响应线(表明新陈代谢活动完全被抑制)。当生理盐水中 Ag NPs 浓度≥24.0 mg/L 时, 在该培养时间范围内没有反正弦型细菌生长曲线出现, 根据国际通用规则(Theophel *et al*, 2014), 可以认为在本实验条件下 Ag NPs 对副溶血弧菌的 MIC 值为 24.0 mg/L。

为了验证 EMGA 法测定纳米材料抑菌活性的重现性和精密度, 在一周内研究团队 3 名成员采用完全相同的操作方法分别进行了 3 次实验, 测定了 Ag NPs 在河口水样品 S1 中对副溶血弧菌的 MIC 值, 得到的 MIC 值皆为 24.0 mg/L。该结果说明 EMGA 法具有良好的精密度和重现性。

作为对比, 实验采用 BMD 法和平板计数法测定

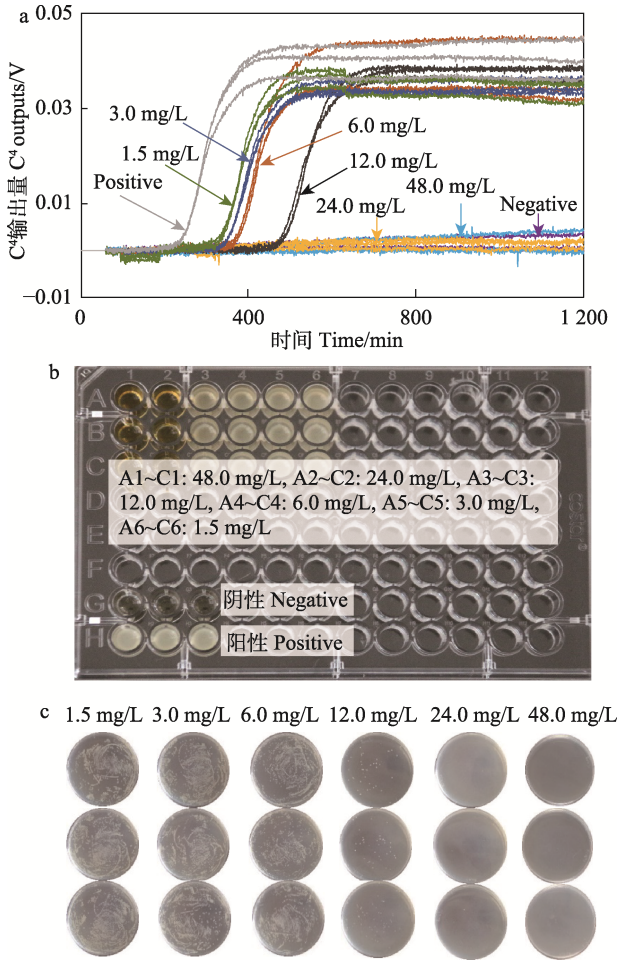

图 3 EMGA 法(a)、BMD 法(b)和平板计数法(c)测定 Ag NPs 在河口水中对副溶血弧菌的抑制作用

Fig.3 Effects of Ag NPs on *V. parahaemolyticus* in estuarine water determined with the EMGA (a), BMD (b) and plate counting (c) methods in triplicate

了相同条件下 Ag NPs 对副溶血弧菌的抑制效应，结果分别见图 3b 和图 3c。由图 3 可知，BMD 法和平板计数法测定的 MIC 值也为 24.0 mg/L，说明采用 EMGA 法分析河口水中 Ag NPs 的抑菌效应时，所得数据和采用经典的 BMD 法和平板计数法所得数据具有良好的一致性。

2.3 EMGA 法性能分析

实验采用 EMGA 法、BMD 法和平板计数法分别测定了 S1、S2、S3、S4、S5、S6、S7 和 S8 共 8 个河口水样品中 Ag NPs 对副溶血弧菌的急性暴露影响，MIC 值如表 2 所示。结果表明，EMGA 法测定结果和 BMD 与平板计数法测定的 MIC 值吻合性很好，基本符合(EA)达到 75%；小偏差(mE)为 25%，未发现大偏差(ME)，说明 EMGA 法测定 Ag NPs 对副溶血弧菌抑菌效应具有良好的准确性。此外，EMGA 法测定的 MIC 值往往大于或等于 BMD 法和平板计数法的测定结果，其原因在于自动化仪器的灵敏度高于肉眼观察。因此，EMGA 法的抑菌效应测定结论比基于肉眼判断的结果具有更高的可靠性。

Zhang 等(2021)研究表明，采用 OD 法测定模拟河口水中纳米材料对大肠杆菌(*Escherichia coli*)的抑制效应时，细菌生长 OD-*t* 曲线因受共存物质光学信号的干扰而波动严重，导致无法读取 MIC 的准确值。本研究采用 BMD 法和平板计数法测定河口水样中 Ag NPs 的抑菌效应时也需要采用离心法除去悬浮颗粒等干扰物，否则严重影响 MIC 值的准确读取(如图 4 所示)。

由于 MIC 值和存活微生物的培养时间具有相关性(Theophel *et al*, 2014)，研究中培养时间全部采用 20 h。在这种情况下，如图 5 所示，采用 EMGA 法测定河口水样中 Ag NPs 对副溶血弧菌的影响时仅需要两步手工操作——将暴露后的混合物直接转移到检测管和上机测定存活细菌的生长动力学曲线，检测总周期为 1 210 min。而采用 BMD 法和平板计数法都需要 3 个步骤，检测总周期为 1 230 min。较之于经典的方法，EMGA 法需要的手工操作步骤较少，意味着耗时、劳动力、费用和误差的降低，因此具有显著的性能优势。

表 2 EMGA 法、BMD 法和平板计数法测定的 MIC 值

Tab. 2 MIC values of Ag NPs against *V. parahaemolyticus* obtained by EMGA, BMD and plate counting methods/(mg/L)

方法 Method	样品 Sample							
	S1	S2	S3	S4	S5	S6	S7	S8
EMGA 法	24.0	—	—	—	—	—	48.0	—
BMD 法	24.0	—	48.0	48.0	—	—	48.0	—
平板计数法	24.0	—	—	48.0	—	48.0	48.0	—

注：—表示 Ag NPs 在 0~48.0 mg/L 范围内未抑制住细菌的生长。

Note: —indicates that Ag NPs did not inhibit the bacterial growth over the concentration range of 0–48.0 mg/L.

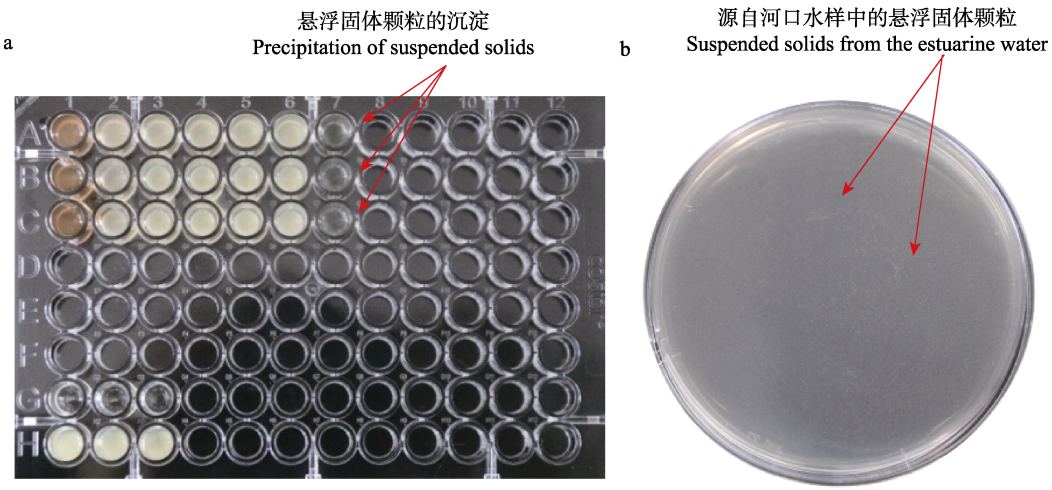

图 4　未去除悬浮固体情况下河口水的 BMD (a)和平板计数(b)结果图像

Fig.4　BMD (a) and plate counting (b) images of estuarine water in case of without removing suspended solids

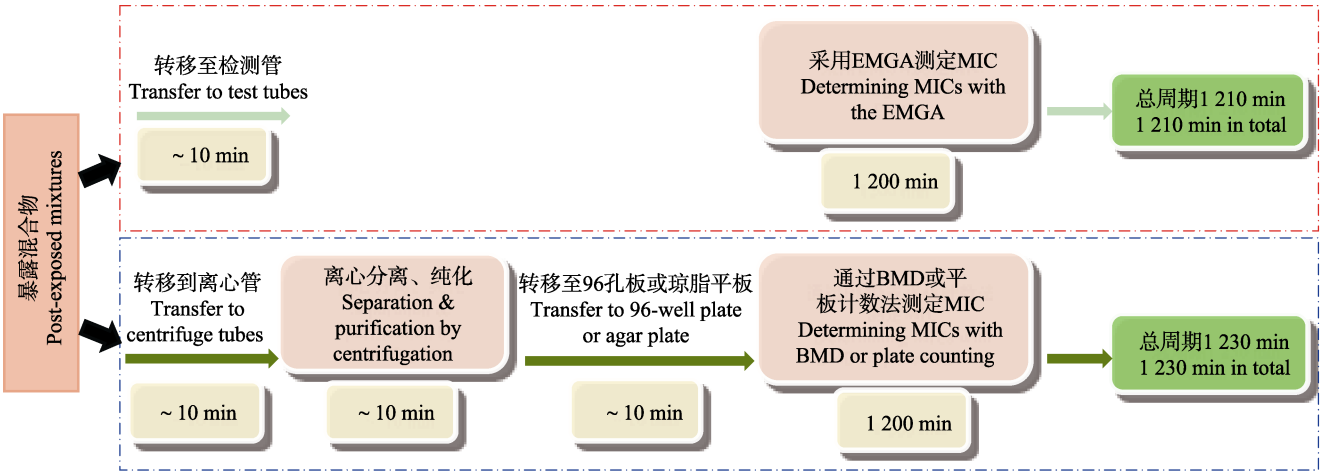

图 5　EMGA 法(上)和 BMD、平板计数法(下)测定 Ag NPs 在河口水中抑菌效应的流程和时效示意图

Fig.5　Schematics of the procedures and time-consumption of determining MICs of Ag NPs against *V. parahaemolyticus* in estuarine water using the EMGA method (upper) and BMD and plate counting methods (lower)

3　结论

本研究建立了一种测定 Ag NPs 在河口水中抑菌效应的表型方法。该方法仅需要两步手工操作：1)直接将暴露混合液加入预装有 LB 液体培养基的检测管中；2)把检测管置入 EMGA 工作通道中测定存活细菌的生长动力学曲线。根据细菌生长曲线形状即可判读出该纳米材料在河口水中对副溶血弧菌的 MIC 值。较之于经典的测定方法(如 BMD 和平板计数)，其优势在于无需去除暴露混合液中诸如悬浮颗粒之类的复杂共存物，因此操作更简便、劳动强度小，总周期缩短至少 20 min。此外，由于手工操作步骤较少，有效地降低了主观和客观误差风险。EMGA 法测得的 MIC 值具有良好的精密度和重现性。同时，该基于传感器识别结果的自动化方法比 BMD 法和平板计数法具有更高的灵敏度。本研究为准确、高效地测定纳米材料在诸如河口水之类复杂介质中的环境毒理效应提供了新手段。

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(编辑　马璀艳)

An Efficient Analytical Method for Determining the Effects of Silver Nanoparticles on *Vibrio parahaemolyticus* in Estuarine Water

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Abstract　In virtue of their distinctive antimicrobial properties, silver nanoparticles (Ag NPs) are some of the most commonly used nanomaterials in the world, with applications in medical equipment, cosmetics, textiles, electronics, toys, and household appliances. As a result, they inevitably end up in rivers, lakes, estuaries, and coastal waters via wastewater, atmospheric deposition, and other pathways. Recent explorations have increased concerns regarding their adverse effects on the ecological health of estuarine environments. For risk assessment of nanomaterials in estuarine environments, microorganisms - especially bacteria - are ideal candidates as bioreporters. Reliable and effective methods for determining the effects of nanomaterials on microorganisms are of significance for assessing ecotoxicities. Growth curve-based methods are popular because they can fully reflect the toxicity of nanomaterials. Genotypic methods, which are based on DNA analysis, provide attractive alternatives. These phenotypic and genotypic methods have performed well in determining the effects of nanomaterials on microorganisms in simple laboratory media. However, when they are used in realistic matrices, such as estuarine water, which is complex in physical, chemical, and ecological characteristics, pretreatment steps for separation and purification are unavoidably applied prior to the determination steps. These pretreatment steps usually pose the risk of subjective and objective errors and poor efficiency. To date, more efficient and accurate analytical methods are still needed for assessing the ecotoxicity of nanomaterials.

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Recently, our research group contributed to an alternative concept for online monitoring of microbial growth by developing a multichannel capacitively coupled contactless conductivity (C^4) detector. C^4 detection is a particular type of conductivity-based analytical method, where the electrodes are not in direct contact with the tested medium. The magnitude of the detected signal (C^4 output) is proportional to the concentration and mobility of the ionic charge carriers within the medium. It not only shares the advantages of common electrochemical techniques, such as instrumental simplicity, affordability, rapid response, nontransparent requirement, and easy miniaturization, but is also free of polarization, passivation, and fouling risks. Based on a 32-channel C^4 detector and special algorithms, we developed a 32-channel electronic microbial growth analyzer (EMGA). EMGA could determine repeatable bacterial growth curves with a high temporal resolution in both homogeneous simple laboratory mediums and heterogeneous matrices.

The EMGA method was used to evaluate the antibacterial effects of Ag NPs on *Vibrio parahaemolyticus*, compared with the use of the broth microdilution method (BMD） and plate counting methods. The minimum inhibitory concentration (MIC) of Ag NPs against *V. parahaemolyticus* in estuarine water samples determined by using the EMGA method is 24.0 mg/L, which is consistent with the results obtained by using the BMD and plate counting methods. The results obtained by using the EMGA method are in good agreement with the MIC values of the BMD and plate counting methods, with an essential agreement (EA) of 75% and minor error (mE) of 25%. No major error (ME) was found, indicating that the EMGA method for measuring the effects of Ag NPs on *V. parahaemolyticus* in estuarine water samples is reliable. In addition, the MIC values obtained by using the EMGA method are often higher than or equal to the results obtained by using the BMD and plate counting methods, due to the higher sensitivity of automated instruments compared to visual observation. Therefore, the antibacterial activity obtained by using the EMGA method is reliable than the results based on visual judgment.

This study established a phenotypic method for determining the antibacterial activity of Ag NPs against *V. parahaemolyticus* in estuarine water. This method requires only two manual steps rather than three as in classical methods such as BMD and plate counting. In addition, due to the elimination of complex coexistent substances, it effectively reduces the risk of subjective and objective operational errors. This automated method based on sensor recognition results has higher sensitivity compared with the BMD and plate counting methods. Thus, the newly proposed method has the advantages of simplicity, time-saving, low-labor intensive, greater precision, and good repeatability. In addition, the sensitivity of this automatic instrument-based method is higher compared with eye-based methods. This efficient method provides a new approach for assessing ecotoxicity of nanomaterials in realistic environmental matrices, such as estuarine water.

Key words Silver nanoparticles; Ecotoxicity; Estuarine water; *Vibrio parahaemolyticus*; Electronic microbial growth analyzer