



UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE BIOTECNOLOGIA
PÓS-GRADUAÇÃO EM GENÉTICA E BIOQUÍMICA

Biossensores eletroquímicos para detecção de HIV

Aluno: Anna Clara Rios Moço

Orientador: Prof^a. Dr^a. Ana Graci Brito Madurro

Co-Orientador: Prof. Dr. João Marcos Madurro

**UBERLÂNDIA-MG
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Biossensores eletroquímicos para detecção de HIV

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Ana Graci Brito Madurro

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LISTA DE ABREVIATURAS

4-ATP: 4-aminotiofenol (*4-aminothiophenol*)
AC: Corrente Alternada (*Alternating Current*)
AFM: Microscopia de Força Atômica (*Atomic Force Microscopy*)
AIDS: Síndrome de Imunodeficiência Adquirida (*Acquired Immunodeficiency Syndrome*)
AM: Azul de Metileno
ART: Terapia Antirretroviral (*Antiretroviral Therapy*)
AuNps: *Gold Nanoparticles*
CCR5: Receptor Quimiocina tipo 5 (*Chemokine Receptor 5*)
CD4: Grupamento de Diferenciação 4 (*Cluster of Differentiation 4*)
C_{DL}: Capacitância da Dupla Camada Elétrica (*Capacitance of Double electric Layer*)
COVID19: *Coronavirus Disease 2019*
CPE: Eletrodo de Pasta de Carbono (*Carbon Pasta Electrode*)
CSPE: Eletrodos impressos de carbon (*Carbon Screen-Printed Electrode*)
CV: Voltametria Cíclica (*Cyclic Voltammetry*)
CXCR4: Receptor Quimiocina tipo 5 (***Chemokine Receptor 4***)
DC: Corrente Contínua (*Direct Current*)
DFM: Microscopia de Força Dinâmica (*Dynamic Force Microscope*)
DLR: Faixa linear de resposta (*Detection Linear Range*)
DNA: Ácido Desoxirribonucleico (*Deoxyribonucleic acid*)
DPV: Voltametria de Pulso Diferencial (*Differential Pulse Voltammetry*)
EB: Brometo de Etídio (*Ethidium Bromide*)
EIS: Espectroscopia de Impedância Eletroquímica (*Electrochemical Impedance Spectroscopy*)
ELISA: Ensaio de Imunoabsorção Enzimática (*Enzyme-Linked Immunosorbent Assay*)
FT-IR: Infravermelho por transformada de Fourier (*Fourier Transform Infrared*)
GCE: Eletrodo de carbono vítreo (*Glassy Carbon Electrode*)

GO: Óxido de grafeno (*Graphene Oxide*)

GoQDs: Pontos quânticos de óxido de grafeno (*Graphene Oxide Quantum Dots*)

GOx: Glicose Oxidase

GQDs: Pontos quânticos de grafeno (*Graphene Quantum Dots*)

gRNA: RNA genômico (genomic RNA)

HIV: Vírus da Imunodeficiência Humana (*Human Immunodeficiency Virus*)

LD: Limite de detecção (Limit of Detection)

MEV: Microscopia Eletrônica de Varredura (*Scanning Electron Microscope*)

MiRNA: micro RNA

MWCNTs: Nanotubos de carbono multicamadas (*Multi-Walled Carbon Nanotubes*)

PEP: Pós-exposição

PrEP: Pré-exposição

PVC: Cloreto de polivinil (*Polyvinyl Chloride*)

QDs: Pontos Quânticos (Quantum Dots)

Rct: Resistência de transferência de carga (*Charge Transfer Resistance*)

rGO: Óxido de grafeno reduzido (*Reduced Graphene Oxide*)

RNA: Ácido ribonucleico (*Ribonucleic Acid*)

Rs: Resistência da Solução (*Solution Resistance*)

RT-PCR: Reação em cadeia da polimerase com transcrição reversa (*Reverse Transcription Polymerase Chain Reaction*)

SELEX: Evolução Sistemática de Ligantes por Enriquecimento (*Exponential Systematic Evolution of Ligands by EXponential Enrichment*)

SPCE: Eletrodos impressos de carbono (*Screen-Printed Carbon Electrodes*)

SPE: Eletrodos impressos (*Screen-Printed Electrodes*)

SPR: Ressonância plasmônica de superfície (*Surface Plasmon Resonance*)

ssDNA: DNA dupla fita (*double-strand DNA*)

ssDNA: DNA simples fita (*single-strand DNA*)

SWV: Voltametria de onda quadrada (*Square Wave Voltammetry*)

TE: Teste Imunoenzimático

TM: Teste Molecular

UNAIDS - Joint United Nations Programme on HIV and AIDS

UV-vis: Ultravioleta visível (*Ultraviolet–Visible*)

Zw: Impedância de Warburg

β -CD: β -ciclodextrina

APRESENTAÇÃO

A infecção por vírus HIV e o desenvolvimento da AIDS (Síndrome de Imunodeficiência adquirida) foram descobertos há mais de 40 anos e, desde então, é uma preocupação global. Ao longo dos anos, o desenvolvimento de antirretrovirais revolucionou o tratamento da AIDS, aumentando a qualidade e expectativa de vida das pessoas que vivem com HIV.

O tratamento da AIDS, além do aumento da qualidade de vida, ocasiona uma diminuição da carga viral tão significativa que o vírus pode ser indetectável e não mais transmissível. Dessa forma, o sucesso do tratamento depende da detecção precoce do vírus e do monitoramento contínuo da carga viral das pessoas que vivem com HIV. Os métodos convencionais de detecção são muito eficientes, porém são caros, necessitam de mão-de-obra especializada, utilização de grande volume de reagentes e não são portáteis. Os biossensores são dispositivos que preenchem essas lacunas e possuem grande potencial para diagnósticos específicos, seletivos, sensíveis, portáteis e estáveis.

Os biossensores eletroquímicos são extensamente explorados no diagnóstico de doenças devido ao seu baixo custo, sensibilidade e rapidez de resposta. Além disso, a nanotecnologia revolucionou o desenvolvimento de biossensores com a aplicação de uma vasta gama de nanomateriais que aumentam a performance desses dispositivos.

Nesse contexto, o presente trabalho objetivou o desenvolvimento de biossensores eletroquímicos em eletrodos impressos artesanais com tinta de carbono modificados com 4-aminotiofenol, nanopartículas de ouro e quantum dots de grafeno funcionalizados com L-cisteína para detecção de RNA genômico de HIV. O trabalho seguiu as normas do programa de Pós-graduação em Genética e Bioquímica e foi dividido em três capítulos. O Capítulo 1 (Fundamentação Teórica) elucida os conceitos teóricos, o Capítulo 2 (*Carbon ink-based electrodes modified with nanocomposite as a platform for electrochemical detection of HIV RNA*) traz o manuscrito publicado na revista *Microchemical* (Anexo 1) e o Capítulo 3 (*High performance label-free genosensor based on a carbon screen printed electrode*

modified with graphene quantum dots and gold nanoparticles for HIV RNA detection) apresenta o segundo manuscrito nas normas da revista *Sensors & Actuators, B: Chemical*

CAPÍTULO 1

FUNDAMENTAÇÃO TEÓRICA

1. Características do HIV

Os primeiros relatos de uma infecção que causava imunodeficiência celular severa com etiologia desconhecida foram reportados em 1981 (MASUR *et al.*, 1981). Essa doença foi conhecida, então, como Síndrome de Imunodeficiência Adquirida (SIDA ou, do inglês, AIDS). Foi em 1983 que o isolamento e identificação do agente causador da AIDS foi realizado e nomeado de Vírus da Imunodeficiência Humana (VIH ou, do inglês, HIV) (BARRE-SINOUSSE *et al.*, 1983).

O HIV é um lentivírus da família *Retroviridae* cujos integrantes compartilham da característica de possuir um genoma de RNA que é utilizado como molde para a síntese de DNA dupla-fita (PAYNE, 2017a). Esse processo é mediado pela enzima viral transcriptase reversa (Figura 1) que é dependente da DNA polimerase. A dupla-fita de DNA resultante se integra ao genoma do hospedeiro por outra enzima, a integrase viral (PAYNE, 2017a).

O genoma do HIV é composto por duas cópias idênticas de RNA fita-simples (Figura 1) com 9.2 kb de tamanho contém os genes estruturais *gag*, *pol* e *env* (SIERRA; KUPFER; KAISER, 2005). O gene *gag* codifica as proteínas estruturais p24, p7 e p6, bem como a proteína de matriz p17. O gene *env* codifica as glicoproteínas do envelope viral gp120 e gp41. As enzimas relacionadas à replicação viral, citadas anteriormente, são codificadas pelo gene *pol*. Essa estruturas podem ser visualizadas na ilustração da Figura 1.

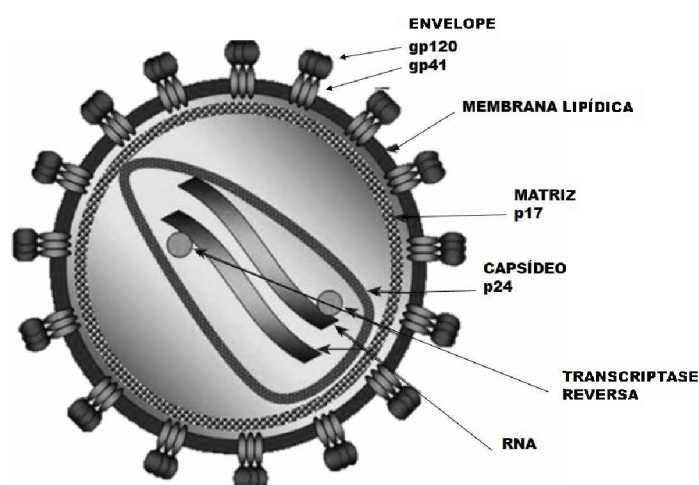


Figura 1. Estrutura da partícula de HIV. Adaptado de Fanales-Belasio *et al.* (2010).

Existem dois tipos de vírus que podem infectar os humanos, o HIV-1 e o HIV-2. O primeiro está relacionado aos casos de infecções globais e o segundo está restrito em algumas regiões da África (FANALES-BELASIO *et al.*, 2010). Os dois tipos são similares quanto aos genes principais da formação da partícula viral, mas diferem na composição das proteínas acessórias (PAYNE, 2017b).

O alvo do HIV é o receptor CD4 presente nas células do sistema imune como linfócitos T auxiliares, macrófagos e células dendríticas (PAYNE, 2017b). O vírus interage com esse receptor via glicoproteína gp120. Após alterações conformacionais, outros receptores, os correceptores CCR5 ou CXCR4 são ativados e o envelope viral se funde à membrana celular por endocitose (SIERRA; KUPFER; KAISER, 2005). Essa fusão permite que o interior do vírus seja liberado no citoplasma da célula infectada. Posteriormente, ocorre a transcrição reversa do RNA do vírus, produzindo o DNA dupla-fita que será integrado ao genoma do hospedeiro pela enzima integrase (PAYNE, 2017a). Novas partículas virais são produzidas e montadas no interior da célula e, quando são liberadas, ocorre lise e morte celular (PAYNE, 2017b).

1.1 Curso natural da infecção

A principal característica para o desenvolvimento da AIDS é a progressiva queda das células T CD4+. Dessa maneira, a infecção se caracteriza pela sua cronicidade e possui três fases em seu curso (Figura 2): fase inicial ou primária, fase assintomática ou de latência clínica e fase sintomática (FANALES-BELASIO *et al.*, 2010).

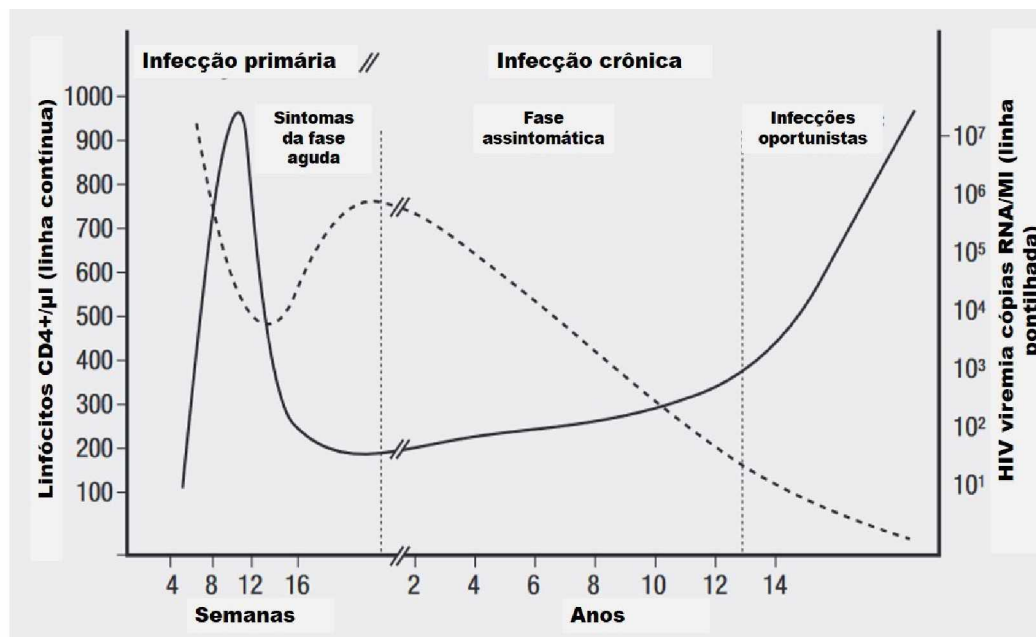


Figura 2. Estágios da evolução da infecção por HIV. Adaptado de Fanales-Belasio *et al.* (2010).

A fase aguda e primária corresponde ao tempo entre o início da infecção e o desenvolvimento da resposta imunológica ou soroconversão. Essa soroconversão ocorre entre 3 a 5 semanas depois da exposição ao vírus (FANALES-BELASIO *et al.*, 2010). Nesse período, ocorre intensa queda das células T CD4+ e alta viremia. Alguns sintomas agudos podem aparecer como febre, mal-estar geral, diarreia e outros semelhantes à gripe (MELHUISE; LEWTHWAITE, 2018).

Após a soroconversão, a fase assintomática se inicia e pode durar de 8 a 12 anos (FANALES-BELASIO *et al.*, 2010). A quantidade de células T CD4+ aumenta, porém não retornam a um valor normal e a viremia se torna constante (GHOSH *et al.*, 2018).

Finalmente, na fase sintomática, a replicação viral é aumentada e a resposta imunológica é diminuída, abrindo espaço para ocorrência de infecções oportunistas por bactérias, vírus, fungos e parasitas (GHOSH *et al.*, 2018).

1.2 Epidemiologia, transmissão e tratamento

No contexto global, segundo a UNAIDS, desde o começo da epidemia de HIV, 84,2 milhões de pessoas foram infectadas com HIV e, em 2021, estima-se que 38,4 milhões de pessoas vivem com HIV. O número de pessoas que foram infectadas no mundo em 2021 é de 1,5 milhões (UNAIDS, 2022). No Brasil, de 2007 até junho de 2022, foram notificados 434 803 casos de infecção por HIV, sendo 183 901 (42,3%) na região Sudeste, 89 988 (20,7%) na região Nordeste, 84 242 (19,4%) na região Sul, 42 957 (9,9%) na região Norte e 33 715 (7,7%) na região Centro-Oeste (B. SAÚDE, 2022).

Os números epidemiológicos mostram que, apesar dos grandes avanços no tratamento da AIDS, a epidemia de HIV é ainda uma preocupação mundial, principalmente em países com recursos limitados (PUGH *et al.*, 2022). Além disso, desde 2019 o mundo enfrenta a pandemia de COVID19 que ocasionou impactos graves em diversas esferas, principalmente na saúde pública mundial (KRITTANAWONG *et al.*, 2022). Em face dessa pandemia sem precedentes, algumas consequências atingiram os esforços no combate ao HIV, como diminuição do acesso aos serviços de saúde e diagnósticos (BARAL *et al.*, 2021).

A transmissão do HIV ocorre, principalmente, por vias sexuais com o contato com fluidos vaginais ou sêmen (MELO *et al.*, 2019). Outras formas de transmissão são: via materna, durante a gravidez, por contato com o sangue de pessoas infectadas e leite materno (CHEN *et al.*, 2019).

No começo da pandemia de HIV muitas pessoas morreram e, por um tempo, a infecção era sinônimo de sentença de morte. Após grandes avanços na ciência, as drogas antirretrovirais revolucionaram o tratamento da AIDS, levando ao aumento da expectativa de vida e fortalecimento imunológico das pessoas que vivem com HIV (SCHINAZI; PATEL; EHTESHAMI, 2022). Esse tratamento é composto pela combinação de drogas antivirais, também conhecido por terapia com *cocktail*, que visa a diminuição da carga viral por diversos mecanismos, como a inativação das enzimas transcriptase reversa, integrase, proteases e outras relacionadas à replicação viral (SCHINAZI; PATEL; EHTESHAMI, 2022).

O início do tratamento deve ser o mais cedo possível, de forma a mitigar os efeitos da infecção, ou seja, quanto mais cedo é a detecção do vírus, mais rápida é a iniciação do tratamento e maior a chance na melhora da qualidade de vida (BOR

et al., 2021). Adicionalmente, o tratamento também faz parte da prevenção da doença, uma vez que os antirretrovirais podem diminuir a carga viral ao nível de o HIV ser indetectável e não mais transmissível (BOR *et al.*, 2021). Outros tipos de prevenção envolvendo as drogas antirretrovirais são preconizados: a profilaxia de pré-exposição (PrEP) e de pós-exposição (PEP) (PHANUPHAK; GULICK, 2020). A PrEP é recomendada para pessoas que têm riscos substanciais de infecção por HIV, como pessoas em relacionamentos sorodiscordantes, utilização inadequada e inconstante de camisinhas ou compartilhamento de injeções. A PEP sexual é indicada até 72h após a exposição ao vírus (PHANUPHAK; GULICK, 2020).

A terapia com antirretrovirais é eficaz em diminuir a viremia e melhorar a qualidade de vida das pessoas que vivem com HIV, indubitavelmente (GHOSH *et al.*, 2018). A cura do HIV é frustrada, principalmente, por uma característica do vírus durante a infecção: a latência viral (SILICIANO; GREENE, 2011). O vírus permanece em estado de latência, sem expressão genética, em células com longo tempo de vida, como as células T CD4+ (KULPA; CHOMONT, 2015). No entanto, quando a terapia antirretroviral é interrompida, ocorre novamente a replicação viral e isso enfatiza a importância da continuidade da terapia (KULPA; CHOMONT, 2015).

1.3 Diagnóstico

O sucesso e o manejo do tratamento da AIDS depende da detecção rápida e precoce do HIV. Após a infecção pelo vírus, alguns biomarcadores específicos aparecem no sangue em cronologias distintas, como pode ser visualizado na Figura 3. O RNA viral aparece depois do décimo dia e pode atingir uma carga viral de mais de 1 milhão de cópias por mililitro (BUSCH; SATTEN, 1997). Alguns dias depois, o antígeno p24 pode ser detectado e sofre um queda devido à complexação desse antígeno com os anticorpos emergentes (FIEBIG *et al.*, 2003). Após cerca de 4 a 6 semanas, os anticorpos começam a aparecer e a maioria das pessoas infectadas são soroconvertidas em 3 meses (MELHUISH; LEWTHWAITE, 2018).

A reatividade de cada teste diagnóstico depende do alvo que será detectado e do tempo de infecção (BRANSON, 2019). Os testes sorológicos detectam antígenos e anticorpos no soro e são, principalmente, os testes imunoenzimáticos

(TE), como o teste ELISA (do inglês *enzyme-linked immunosorbent assay*) (BRASIL, 2018). Os testes moleculares (TM) são utilizados para detecção de RNA e DNA, como a RT-PCR (do inglês *reverse transcription polymerase chain reaction*) (BRASIL, 2018).

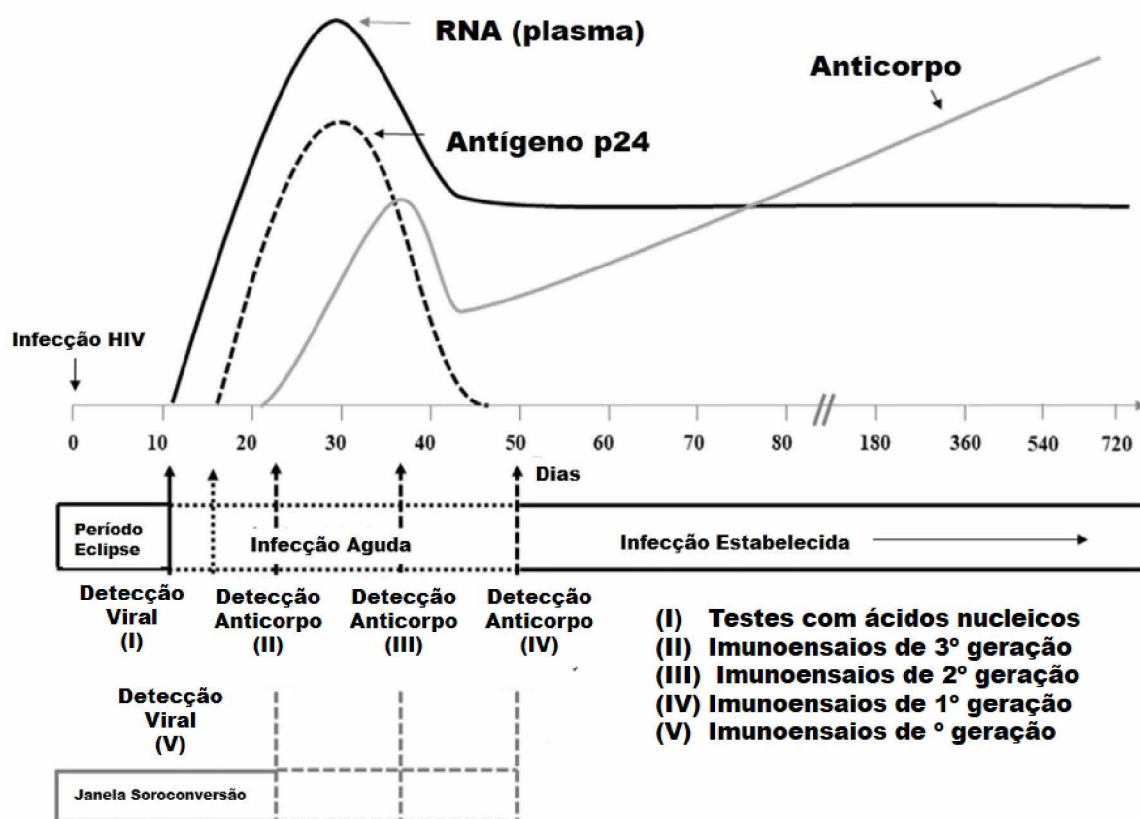


Figura 3. Tempo de infecção e surgimento dos biomarcadores para HIV. Adaptado de Bronson (2019).

Os testes IE e moleculares são utilizados para além da detecção do HIV, sendo aplicados para determinar o status da infecção, avaliar a progressão da doença, analisar a latência viral e monitorar a eficácia da terapia antirretroviral (PHANUPHAK; GULICK, 2020; SILICIANO; GREENE, 2011). As ferramentas utilizadas para detecção e/ou diagnóstico podem ser divididas em dois ensaios: quantitativo e qualitativo. O primeiro é utilizado para avaliar a progressão clínica da infecção, bem como a análise da carga viral. A segunda pode ser conduzida com ferramenta diagnóstica da infecção por HIV (ZHAO; CHANG; WANG, 2019).

Os testes ELISA e RT-PCR são considerados padrão ouro no diagnóstico do HIV, porém apresentam limitações no que tange a utilização em campo e em países com recursos limitados, tais como: necessidade de pessoal qualificado e equipamentos robustos, várias etapas na execução, grande volume de reagentes e custo elevado (BUKASOV; DOSSYM; FILCHAKOVA, 2021). Nesse sentido, vários outros métodos diagnósticos são estudados e desenvolvidos objetivando as características de um diagnóstico ideal, a saber: sensibilidade e especificidade alta, custo razoável, facilidade no manuseio, rápida resposta, tempo de prateleira longo em temperatura ambiente e portabilidade (DESAI; WU; ZAMAN, 2011).

2. Biossensores

2.1 Definição

O biossensor é um dispositivo analítico para mensurar reações biológicas ou químicas através de geração de sinal proporcional à concentração do analito de interesse. O dispositivo é composto pelos seguintes componentes (Figura 4): biorreceptor, transdutor, dispositivo eletrônico e exibição dos dados (KARUNAKARAN; RAJKUMAR; BHARGAVA, 2015). O biorreceptor é a molécula responsável por reconhecer o analito de maneira específica. O analito é a substância de interesse e neste trabalho é referido como alvo (*target*). A interação entre o biorreceptor e o analito causa a geração de um sinal que é detectado pelo transdutor. Assim, o transdutor tem a função de converter uma forma de energia em outra e, nesse caso, a conversão do evento de biorreconhecimento em um sinal mensurável. Esse sinal transduzido é exibido na forma de números, gráficos, tabelas ou imagens (TETYANA; MORGAN SHUMBULA; NJENGELE-TETYANA, 2021).

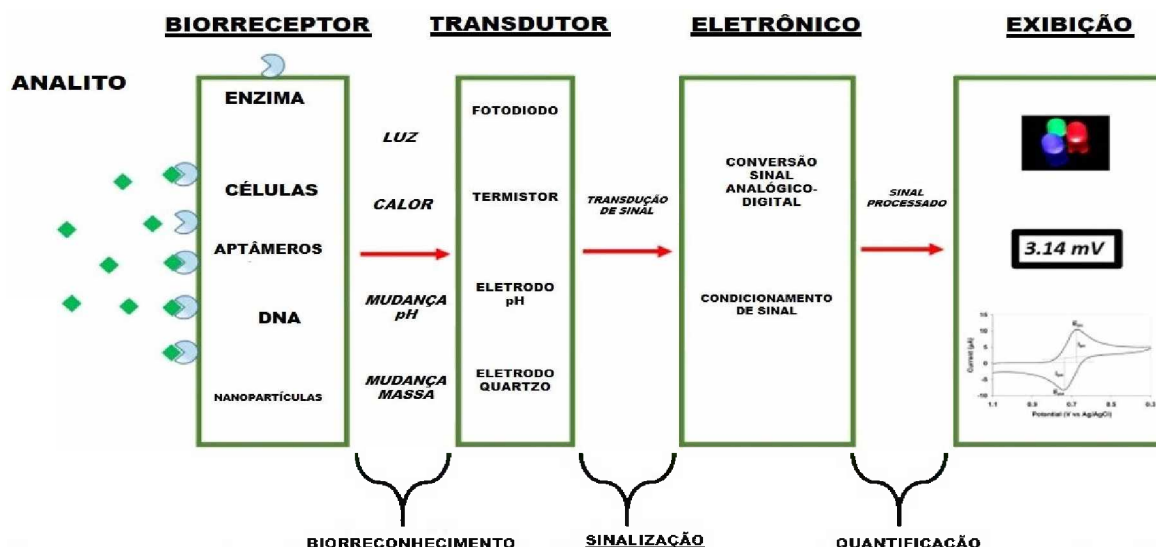


Figura 4. Componentes do biossensor. Adaptado de Karunakaran (2015).

O primeiro biossensor foi desenvolvido em 1956 por Leland C. Clark, conhecido como “pai dos biossensores”. O biossensor de Clark detectava glicose em amostras biológicas utilizando como biorreceptor a enzima glicose oxidase (CLARK, 1956). Desde então, muitas pesquisas foram realizadas com objetivo de desenvolver biossensores altamente sensíveis, seletivos e específicos. Na busca pelo termo “biossensor” na plataforma do *Science Direct* é encontrado 85.526 resultados de artigos de pesquisa entre 1999 e 2023, evidenciando o crescimento exponencial, que é o reflexo da importância do tema.

As aplicações de biossensores são vastas e envolvem soluções para diagnóstico de doenças, biomedicina, segurança alimentar, monitoramento ambiental, descobrimento de drogas e defesa nacional (FLAUZINO *et al.*, 2022; MOÇO *et al.*, 2021; CHIOU *et al.*, 2015; PRÉVEL *et al.*, 2014).

2.2 Classificação

A classificação dos biossensores pode ser dividida sob a perspectiva do biorreceptor e do transdutor. A Figura 5 mostra a classificação sob as duas perspectivas com alguns exemplos que serão detalhados a seguir.

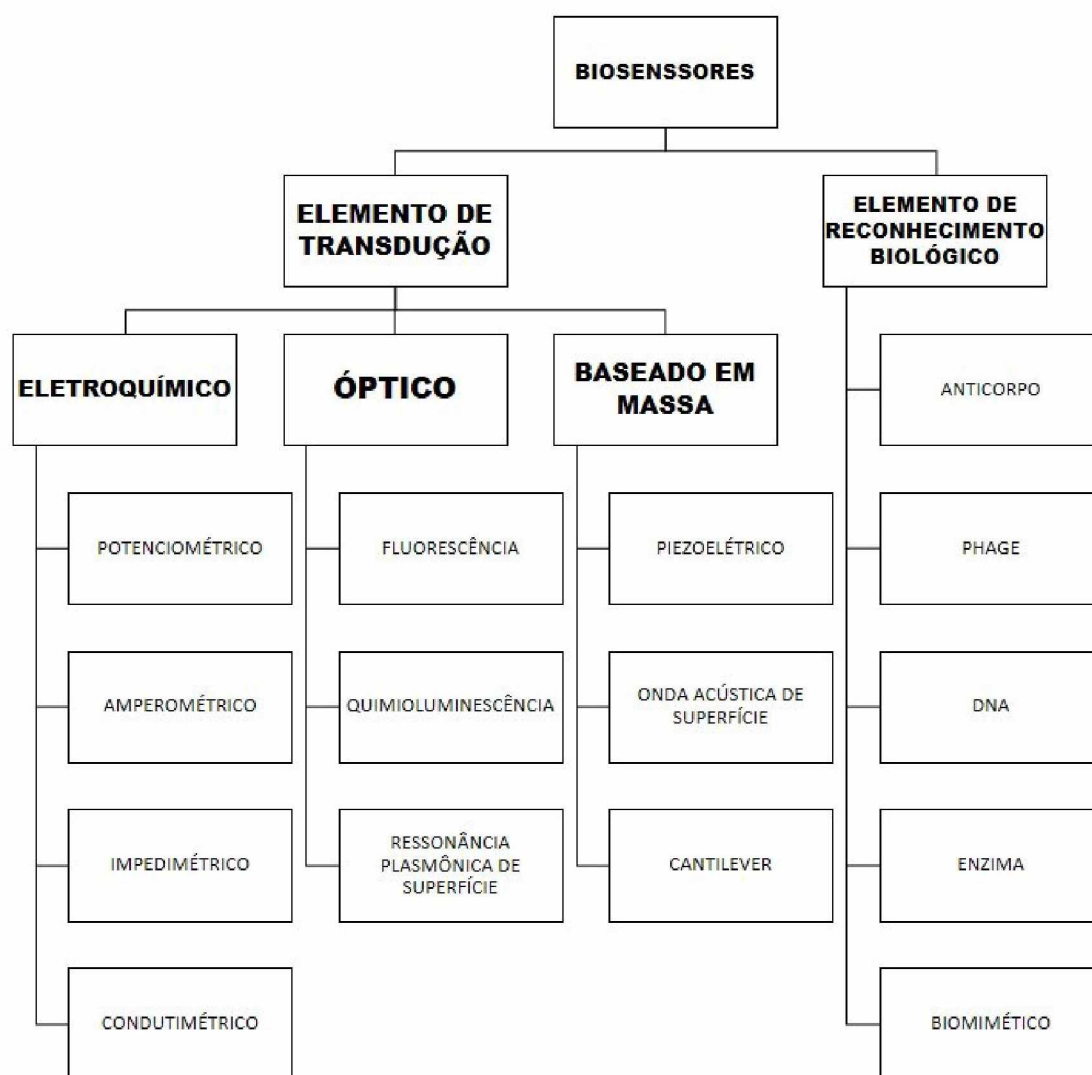


Figura 5. Classificação dos biossensores sob duas perspectivas e exemplos.
 Autoria própria.

2.2.1 Classificação quanto ao biorreceptor

A classificação baseada no biorreceptor caracteriza-se pela biomolécula responsável pelo reconhecimento do analito como enzimas, DNA, anticorpos e células. Os princípios que norteiam o biorreconhecimento por essas biomoléculas serão tratados a seguir.

2.2.1.1 Enzimas

Os biossensores enzimáticos possuem grande apelo comercial e são bem estabelecidos no mercado desde o desenvolvimento do primeiro biossensor de Clark para detecção de glicose (TETYANA; MORGAN SHUMBULA; NJENGELE-TETYANA, 2021). Esse sucesso não é por acaso. As enzimas são biomoléculas com propriedades que garantem a especificidade e seletividade do analito (ROSKOSKI, 2007). Sob condições ótimas, a enzima reconhece um substrato específico em seu sítio ativo e catalisa a reação para formação de produtos que são detectáveis pelo transdutor. No biossensor de glicose, por exemplo, a enzima glicose oxidase (GOx) é imobilizada no eletrodo e catalisa a oxidação de β -D-glicose gerando, ácido glucônico e peróxido de hidrogênio (ZHANG *et al.*, 2021).

2.2.1.2 DNA

A molécula de DNA possui vários atrativos no desenvolvimento de biorreceptores para biossensores de alta performance. Dentre suas características, o princípio de Watson-Crick no pareamento específico das bases nitrogenadas se destaca. Os pequenos fragmentos de DNA (oligonucleotídeos), que são imobilizados em determinada plataforma, conseguem se ligar, por ligações de hidrogênio, ao seu alvo complementar em uma matriz complexa, garantindo seletividade e especificidade (TETYANA; MORGAN SHUMBULA; NJENGELE-TETYANA, 2021).

Além da característica de pareamento de bases, o DNA possui outras características interessantes, como: estabilidade, programabilidade e biocompatibilidade. O DNA consegue se manter estável em meios básicos ou ácidos e pode ser recuperado mesmo após passar por altas temperaturas (WANG *et al.*, 2022). A programabilidade se refere à possibilidade de sintetizar o DNA com diferentes estruturas e dinâmicas, como a recente tecnologia do DNA origami (TADAKUMA; MASUBUCHI; UEDA, 2016). Nessa tecnologia, o DNA assume formas complexas nanoestruturadas. A nanotecnologia do DNA está sendo estudada e será um tópico importante no desenvolvimento de biossensores da próxima geração (RANADE; DATTA, 2023).

2.2.1.3 Anticorpos

A interação entre anticorpos e antígenos é bastante explorada nos métodos imunoenzimáticos, citados no tópico 1.3. Os anticorpos são imunoglobulinas que reconhecem parte dos antígenos através de complementaridade espacial. Quando o anticorpo se liga ao antígeno, se forma um complexo que, por sua vez, é precipitado. Esse complexo é formado por diferentes interações como interação eletrostática e hidrofóbica, ligações de hidrogênio e forças de Van der Waals (ZHANG; ZHAO, 2019). A interação anticorpo-antígeno normalmente é melhorada utilizando anticorpos recombinantes (ALHADRAMI, 2018).

2.2.1.4 Células e micro-organismos

Células inteiras ou micro-organismos, como bactérias e vírus, podem ser utilizados no biorreconhecimento (ALHADRAMI, 2018). Uma grande vantagem é que a interação entre célula e alvo reproduz os efeitos esperados, mimetizando o ambiente natural (ROGGO; VAN DER MEER, 2017). Essas células e micro-organismos vivos também são referidos como biorrepórteres. Os biorrepórteres têm grande potencial no monitoramento de toxinas em amostras ambientais, porém apresentam desvantagens como demora e baixa sensibilidade (ROGGO; VAN DER MEER, 2017).

Os bacteriófagos (*phage*) são vírus que infectam bactérias e são utilizados na detecção de bactérias (FAROOQ et al., 2018). Além disso, os *phages* podem agir como biorrepórteres quando são modificados geneticamente com inserções de genes que podem ser então expressados com características monitoráveis (ALHADRAMI, 2018). Um exemplo disso é a inserção de genes como *lux* e *luc* que geram bioluminescência (FAROOQ et al., 2018).

2.2.2 Classificação quanto ao transdutor

A classificação dos biossensores quanto ao modo de transdução é a mais comumente utilizada. Existem muitos modos de conversão de uma forma de energia em outra, mas essa classe é majoritariamente dividida em três grandes grupos: ópticos, baseados em massa e eletroquímicos (TETYANA; MORGAN SHUMBULA; NJENGELE-TETYANA, 2021). Os próximos tópicos vão tratar desses

tipos de biossensores com maior ênfase nos eletroquímicos, objetivo do presente trabalho.

2.2.2.1 Ópticos

Nos biossensores ópticos, as alterações dos componentes da luz podem ser monitoradas, como o índice de refração, fluorescência, comprimento de onda e absorção. Algumas técnicas utilizadas são: espectrofotometria, fluorimetria, quimioluminescência e ressonância plasmônica de superfície (SPR) (ONG *et al.*, 2021).

A tecnologia de SPR é amplamente utilizada, principalmente pelo seu potencial de análise em tempo real e possibilidade de detecção sem utilização de marcadores nas biomoléculas para mensuração de sinal, característica esta conhecida como *label-free* (CARRASCOSA; HUERTAS; LECHUGA, 2016). O princípio do SPR baseia-se na mudança no índice de refração ocasionado pelas interações moleculares na superfície de um metal, produzindo diferentes ondas plasmônicas na superfície. Esse fenômeno acontece quando os fótons da onda incidente interagem com os elétrons do metal na superfície (CHEN; WANG, 2020). Quando o plasmon de superfície é confinado em nanopartículas, ocorre a oscilação coletiva de elétrons livres, uma vez que o comprimento de onda incidente tem dimensões similares ao tamanho dessas nanopartículas (CHEN; WANG, 2020). Nesse caso, os métodos ópticos podem monitorar essa oscilação coletiva, conhecida por plasmon de superfície localizado (LSP).

2.2.2.2 Baseados em massa

Os biossensores baseados em massa são representados pelas plataformas piezoelétricas. Os materiais piezoelétricos podem sofrer deformação que, por sua vez, provoca polarização elétrica (REGTIEN; DERTIEN, 2018).

A microbalança de cristal quartzo é utilizada em biossensores e monitora a mudança na frequência de um ressonador de quartzo; finos discos de quartzo com eletrodos metálicos em lados opostos são utilizados e, quando uma corrente é

aplicada, geram a ressonância em determinada frequência, que é inversamente proporcional à espessura do material (TUANTRANONT et al., 2011).

2.2.2.3 Eletroquímicos

A eletroquímica estuda as interações entre diferentes materiais que conduzem eletricidade. O grande foco dessa área é estudar as zonas de interface elétrica entre materiais e reações de oxidação e redução. As investigações das reações analisam a geração de uma corrente elétrica, potencial, diferença de cargas, indução de condutividade e análise de impedância (CLARKE; D'ORAZIO, 2020). Para cada um desses parâmetros, diferentes técnicas são utilizadas e são divididas em quatro grandes categorias: potenciometria, amperometria (voltametria), condutimetria e técnicas impedimétricas (ZHU; SHI, 2019).

Antes de abordar essas técnicas, é necessário o conhecimento do sistema eletroquímico. As análises eletroquímicas são realizadas, principalmente, em sistemas com três eletrodos, incluindo o eletrodo de trabalho (WE – *Working Electrode*), o contra eletrodo (CE – *Counter Electrode*) e o eletrodo de referência (RE – *Reference Electrode*) (Figura 6) (REZAEI; IRANNEJAD, 2019). As reações que serão analisadas ocorrem no eletrodo de trabalho, que é composto por diferentes tipos de materiais inertes como carbono, ouro, platina e mercúrio. O contra eletrodo ou eletrodo auxiliar forma uma corrente estável com o eletrodo de trabalho, completando o circuito elétrico. O eletrodo de referência possui um potencial conhecido que se mantém constante durante as medidas no eletrodo de trabalho. A solução contendo o analito de interesse fica em contato com o eletrodo de trabalho e desenvolve potenciais dependentes da atividade do analito (REZAEI; IRANNEJAD, 2019).

A miniaturização e portabilidade são requisitos no desenvolvimento de biossensores com potencial mercadológico. Nesse contexto, os três eletrodos supracitados foram reduzidos e estão disponíveis comercialmente (GARCÍA-MIRANDA FERRARI; ROWLEY-NEALE; BANKS, 2021b). A Figura 6 mostra sistemas eletroquímicos na estrutura tradicional (Figura 6A) e miniaturizados (Figura 6B). Os eletrodos impressos comerciais são eletrodos impressos (do inglês, *Screen-Printed Electrodes* – SPEs) por impressoras que depositam o material

desejado em um substrato plano como o PVC e cerâmica (TUDORACHE; BALA, 2007).

As plataformas mais simples e de baixo custo utilizam pastas e tintas para deposição. As pastas contendo grafite como agente condutor não metálico são populares na eletroquímica, oferecendo limites de detecção baixos e, no geral, as pastas de carbono possuem várias vantagens na aplicação em biossensores como a possibilidade de renovação da superfície, economia de fabricação e estabilidade (TUDORACHE; BALA, 2007).

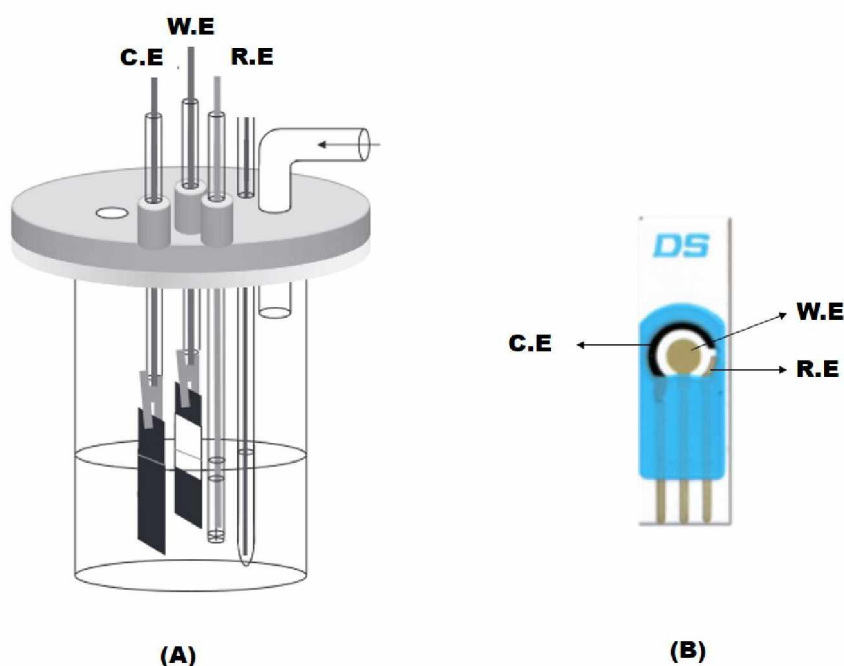


Figura 6. Sistema eletroquímico de três eletrodos. (A) Célula eletroquímica tradicional. (B) Eletrodo impresso comercial. Adaptado de Ueda, Mito e Ohtsuka (2008) e García-Miranda Ferrari, Rowley-Neale e Banks (2021).

2.2.2.3.1 Potenciometria

A potenciometria é definida como a medida do potencial elétrico entre dois eletrodos quando a corrente na célula é zero. A análise da carga do potencial acumulado é realizada no eletrodo de trabalho e comparada ao eletrodo de referência, obtendo dados da atividade de íons na reação eletroquímica (REZAEI;

IRANNEJAD, 2019). Com a corrente negligenciável, a medida de potencial se atribui ao número de espécies reativas presentes na amostra.

O eletrodo de vidro de pH e os eletrodos íon-sensitivos são exemplos de sensores químicos potenciométricos e neles são utilizados dois eletrodos para medida de potencial de uma membrana que reage seletivamente aos íons de determinada amostra (CLARKE; D'ORAZIO, 2020).

2.2.2.3.2 Condutimetria

Os métodos condutimétricos se baseiam na habilidade dos íons em solução apresentarem uma corrente sob potencial constante. Essa corrente é diretamente proporcional à condutividade da amostra. A análise é realizada quando um potencial é aplicado entre dois eletrodos e a corrente é medida; se ocorre um aumento de corrente, há um aumento na condutividade e diminuição da resistência (CLARKE; D'ORAZIO, 2020).

Esse tipo de detecção eletroquímica é utilizado em biossensores para monitoramento ambiental e normalmente possuem enzimas que geram produtos carregados que alteram a força iônica e, conseqüentemente, ocorre um aumento de condutividade (ZHU; SHI, 2019). Outro exemplo de utilização de diferença de condutividade é a medida de hematócritos no sangue, partindo do princípio de que os eritrócitos são isolantes naturais devido a sua membrana lipídica; as células vermelhas são menos condutivas do que o plasma (CLARKE; D'ORAZIO, 2020).

2.2.2.3.3 Técnicas impedimétricas

As técnicas impedimétricas baseiam-se na medida de uma variável física, a impedância. Essa variável analisa as características da resistência de um circuito elétrico na presença de uma corrente alternada (AC) aplicada entre eletrodos (BARD *et al.*, 2002). Esses métodos refletem a natureza da superfície do eletrodo e a transferência eletrônica nessa superfície (REZAEI; IRANNEJAD, 2019).

A lei de resistência de Ohm governa os princípios da impedância, aplicando uma corrente direta (DC) (MACDONALD; JOHNSON, 2005). Quando ocorre a ação de uma AC, o sinal aplicado não é mais estático, como quando se aplica DC, e

oscila como uma onda sinusoidal em determinada frequência. A amplitude dessa onda permanece constante, porém a frequência é variada (BARD *et al.*, 2002).

Em termos da superfície de um eletrodo, a interação eletrodo e íons forma um capacitor na superfície (BARD *et al.*, 2002). Adicionalmente, a interação dos íons de carga oposta com o eletrodo forma a chamada camada dupla eletroquímica. Assim, quando um potencial é aplicado ocorre a transferência de elétrons do analito para a superfície do eletrodo e diferentes impedâncias são analisadas dependendo da composição da superfície e do analito (BARD *et al.*, 2002). Simplificadamente, sob a ação de uma voltagem alternada, ocorrem três processos de oxidação e redução: transferência de massa de íons da solução até a interface do eletrodo, difusão de íons da camada difusa e interação de carga com a camada dupla na interface sólido/líquido do eletrodo de trabalho (CHAKRABORTY; TIBAREWALA; BARUI, 2019).

A impedância é comumente representada por dois gráficos na EIE (Espectroscopia de Impedância Eletroquímica): Bode (Figura 7A) e Nyquist. (Figura 7B). No primeiro, a impedância (Z), plotada versus a frequência (f) e ângulo de fase (θ) se relacionam em dois, sendo a frequência e magnitude da impedância, expressas em uma escala logarítmica. No segundo, a impedância é dividida em real (Z_R) e imaginária (Z_I) (BARD *et al.*, 2002).

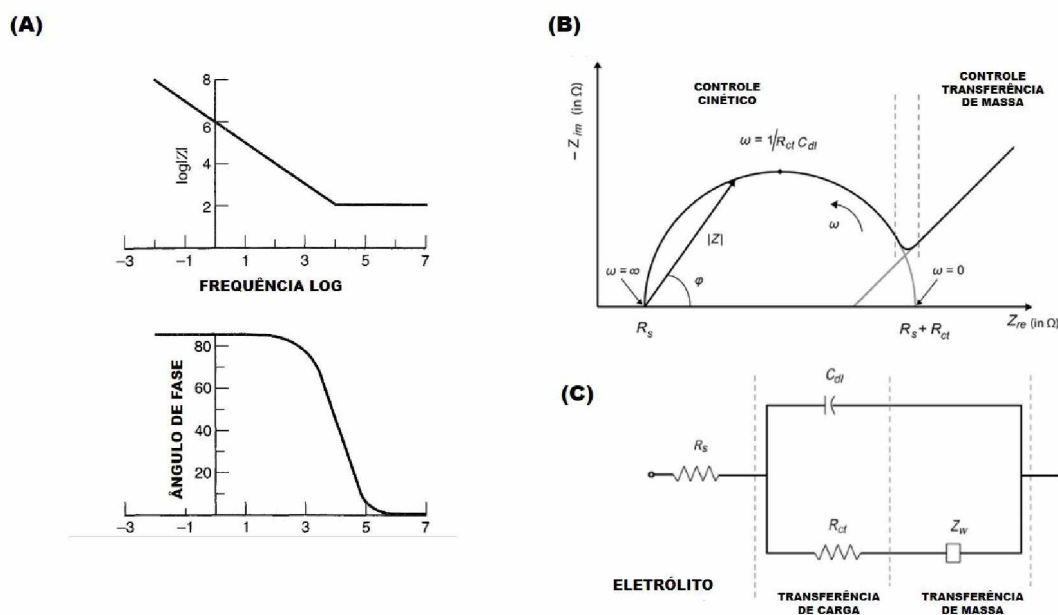


Figura 7. Diagramas de Bode (A) e Nyquist (B) para impedância e circuito de Randles (C). Adaptado de Bard (2002) e Chakraborty, Tibarewala e Barui (2019).

A interface eletrodo/solução pode ser representada como um circuito elétrico equivalente e são importantes ferramentas para análise dos dados experimentais. O circuito de Randles é bastante utilizado para ensaios onde um eletrodo está em contato com um eletrólito e inclui os seguintes componentes (Figura 7C): resistência da solução (R_s), resistência de transferência de carga (R_{ct}), capacitância da dupla camada elétrica (C_{DL}) elemento de transferência de massa (Z_w – impedância de Warburg) (BARD *et al.*, 2002). A R_s e Z_w são constantes depois da interação com os analitos na superfície do eletrodo, porém após um evento de biorreconhecimento do analito, ocorre mudanças em R_{ct} e C_{dl} (CHAKRABORTY; TIBAREWALA; BARUI, 2019)

2.2.2.3.2 Amperometria

Os métodos amperométricos determinam, constantemente, a corrente que resulta da redução ou oxidação de espécies eletroativas na superfície de um eletrodo, ou seja, amperometria mede as variações na corrente através do tempo sob potencial constante (BARD *et al.*, 2002). Na voltametria ocorre a medida da corrente utilizando um potencial variável controlado e a resposta de corrente pode ser observada como picos ou platôs que correspondem à concentração do analito (BARD *et al.*, 2002). O controle de um potencial variável ou fixo ocasiona um excesso de potencial (diferença entre potencial aplicado e de equilíbrio) que torna a transferência eletrônica termodinamicamente favorável, ocorrendo reações de oxidação e redução (ZHU; SHI, 2019).

Esse trabalho foca na voltametria, uma vez que essa técnica corresponde ao principal método eletroquímico dos manuscritos apresentados no Capítulo 2 e 3. Para tanto, é necessária a compreensão dos processos que governam as reações na voltametria e fatores que vão compor esses processos.

Os processos de oxidação e redução e transferência eletrônica têm como princípio a lei de Farady e, conseqüentemente, são chamados de processos

faradaicos. Outros processos que interferem na interface eletrodo-solução, mas não resultam em transferência de cargas, são denominados processos não-faradaicos. Os dois tipos de processos ocorrem nas reações do eletrodo e o processo faradaico normalmente é o desejado, visto que representa a transferência de carga e as reações associadas (BARD *et al.*, 2002).

Os fatores que afetam a taxa de reação e a corrente são: transferência da espécie do corpo da solução para a interface eletrodo-superfície, transporte de massa por difusão e convecção, transferência de elétrons na superfície do eletrodo, reações químicas procedidas de transferência eletrônica e outras reações de interface como adsorção e dessorção (HAMMOND *et al.*, 2016). A transferência de massa se refere ao acúmulo de espécies de um local ao outro na solução que resulta em diferenças no potencial elétrico e químico nesses locais; os modos de transferência de massa podem ser por movimento de uma partícula carregada sob a influência de um campo elétrico (migração), movimento de espécies sob influência de um gradiente de concentração (difusão) e por transporte hidrodinâmico (convecção) (BARD *et al.*, 2002).

Ao levar em consideração os fatores que alteram a corrente, o resultado final será a soma de outras correntes, a saber: residual, de migração e de difusão. A corrente residual é a soma da corrente faradaica (processos redox) e da corrente capacitiva (carga da dupla camada elétrica). A corrente de migração resulta das interações eletrostáticas entre o eletrodo e uma parte do analito no processo de oxidação e redução. Por fim, a corrente de difusão é aquela que é gerada da oxidação ou redução das espécies eletroativas que entraram em contato com o eletrodo por meio do processo de difusão, anteriormente citado (BARD *et al.*, 2002).

São vários os tipos de voltametria e no presente trabalho serão tratadas as voltametrias cíclica, de pulso diferencial e de onda quadrada (Figura 8). Essas são técnicas bastante utilizadas na área de biossensores e algumas diferenças e aplicações serão abordadas a seguir.

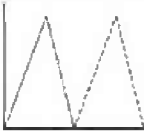
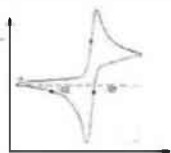
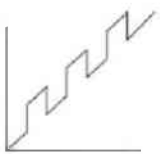
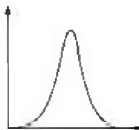
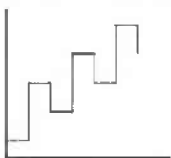
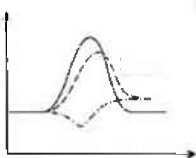
Técnica voltamétrica	Sinal de excitação (E vs t)	Corrente resultante (i vs E)
(A) Voltametria Cíclica		
(B) Pulso Diferencial		
(C) Onda Quadrada		

Figura 8. Técnicas voltamétricas e seus diferentes sinais de excitação e corrente resultante. Autoria própria baseado em Skoog (2005).

Na voltametria cíclica ocorre a aplicação de potencial tanto para regiões anódicas (positivas) quanto para catódicas (negativas) e por isso é chamada de cíclica, ou seja, a varredura de potencial ocorre no sentido direto e inverso. Durante a varredura do potencial, a corrente *versus* o potencial aplicado (I vs E) no eletrodo de trabalho é registrado. As espécies oxidadas geradas no sentido direto são reduzidas no sentido inverso, gerando picos separados para a reação de oxidação e de redução, respectivamente, e cada uma delas caracterizadas por um potencial e corrente de pico. As funções relacionando potencial e tempo (E vs t), chamadas de sinais de excitação, são representadas como ondas pulsadas e triangulares, como pode ser visualizado na Figura 8A e depende do tipo de voltametria (SKOOG, 2005).

A voltametria cíclica é bastante utilizada na caracterização o comportamento redox de moléculas eletroativas, na determinação de processos químicos das reações eletroquímicas na análise da cinética da transferência de elétrons.

A voltametria de pulso diferencial baseia-se na aplicação de uma série de pulsos com amplitude constante que são sobrepostos a uma rampa de potencial crescente. A corrente registrada resultante é uma subtração da corrente aplicada antes (S1) e depois (S2) do pulso. Assim, o voltamograma da corrente final é de forma gaussiana e a concentração do analito é proporcional à área de pico (Figura 8B). Assim, esse tipo de medida é mais sensível do que a voltametria cíclica, uma vez que a subtração das correntes corrige a corrente capacitiva envolvida no processo (SKOOG, 2005).

Na voltametria de onda quadrada, uma onda quadrada simétrica de amplitude ΔE_p , sobreposta a um potencial crescente na forma de escada, com amplitude ΔE_s , largura a e período característicos são aplicados ao eletrodo de trabalho (Figura 8C). Assim como na voltametria de pulso diferencial, a corrente é medida antes e depois da aplicação do pulso, diminuindo influências de correntes não-faradáicas (SKOOG, 2005). Essa técnica é bastante sensível, porém a grande diferença da voltametria de pulso diferencial está na velocidade da detecção, visto que ocorre um aumento da frequência dos ciclos de onda e a aquisição da corrente resultante é mais rápida (BARD *et al.*, 2002).

3. Eletrodos modificados em biossensores

A performance dos biossensores é baseada em parâmetros, como: sensibilidade, seletividade, estabilidade, limite de detecção, tempo de resposta e faixa linear de resposta (TETYANA; MORGAN SHUMBULA; NJENGELE-TETYANA, 2021). A sensibilidade é uma das características mais importantes em biossensores e é definida como a relação entre a mudança de concentração do analito e a intensidade do sinal geral de um transdutor; idealmente, o biossensor deve gerar sinais mesmo em quantidades diminutas de analito. A seletividade se refere à ligação específica com um analito mesmo na presença de diferentes analitos e matrizes; esse parâmetro é crucial no desenvolvimento de biossensores, por exemplo para diagnóstico de doenças, uma vez que envolvem a utilização de sangue, plasma, soro e saliva, por exemplo. A estabilidade avalia a integridade da resposta do biossensor em determinado período de tempo; a integridade da resposta pode ser afetada por fatores externos, como temperatura e umidade. O

limite de detecção se refere à concentração mínima do analito alvo que o biossensor consegue detectar e a faixa linear e resposta é a linearidade do sinal com a variação de diferentes concentrações de analito. Por fim, o tempo de resposta determina o tempo necessário para o biossensor gerar um sinal após a interação entre analito e biorreceptor.

A modificação da superfície de eletrodos é uma abordagem muito utilizada para melhorar e atingir as características ideais de um biossensor (ADARAKATTI; KEMPAHANUMAKKAGARI, 2019). Os eletrodos modificados podem, entre outros exemplos, aumentar a especificidade do biorreconhecimento, aumentar processos redox específicos e ocasionar efeitos sinérgicos que melhoram o efeito catalítico e condutividade, o que acelera a transdução do sinal (ADARAKATTI; KEMPAHANUMAKKAGARI, 2019; EDWARDS; BERGREN; PORTER, 2007; MONTEIRO; JÚNIOR; SUSSUCHI, 2020). Esses efeitos são atingidos, principalmente, por meio da aplicação de nanomateriais e nanocompósitos no desenvolvimento dos biossensores (ZHU *et al.*, 2015). A seguir, serão abordados alguns conceitos desses materiais com enfoque nos nanomateriais utilizados neste trabalho.

3.1 Nanoestruturas e nanocompósitos

A nanotecnologia tem revolucionado o desenvolvimento de biossensores de alta performance (LEE; ADEGOKE; PARK, 2019; SHRESTHA, 2022). As nanopartículas e nanomateriais possuem propriedades únicas, como o tamanho e biocompatibilidade e biodisponibilidade. Muitas características são exploradas devido ao tamanho nano, como as características ópticas e elétricas. Para tanto, é necessário citar a definição de nanomateriais e nanocompositos. A definição de nanomateriais se refere ao tamanho e materiais com diâmetro entre 1 e 100 nm. A classificação depende do movimento de elétrons no nanomaterial: a presença de elétrons é geralmente fixada em dimensão “0”; na dimensão “1”, os elétrons podem se mover no eixo x; nas dimensões 2 e 3, os elétrons se movem melhor do eixo x ao y ou dos eixos x, y, z, respectivamente (KHAN, 2020). O nanocompósito se refere à combinação de diferentes nanomateriais. Muitas combinações são

possíveis, como a combinação de nanomateriais baseados em metal e carbono, bem como materiais poliméricos (ASHA; NARAIN, 2020).

A área da nanotecnologia é bastante explorada e vários nanomateriais são desenvolvidos. Esse trabalho enfoca nas nanopartículas de ouro, aqui referidas como AuNps, e os pontos quânticos ou *quantum dots* (QDs). Todos esses materiais possuem características dependentes do tamanho e abaixo serão abordadas, brevemente, alguns fenômenos que ocorrem devido à mudança de tamanho, como os efeitos quânticos e ressonância plasmônica de superfície.

Os materiais possuem grande diferença quando são reduzidos à escala nanométrica. As características físicas e ópticas mudam, como a área superficial, ponto de fusão, adsorção óptica, solubilidade, carga, entre outros (KHAN, 2020). O aumento da área superficial é uma das principais características que tornam os nanomateriais interessantes. Esse aumento da área superficial por unidade de volume ocasiona uma maior atividade desses materiais quando comparados ao material de origem (*bulk*), principalmente no que tange a interação com outros materiais (ASHA; NARAIN, 2020).

Os efeitos quânticos aparecem em nanoescala e são responsáveis pelas diferentes propriedades dos nanomateriais. O confinamento quântico e a ressonância plasmônica de superfície (SPR) são dois fatores cruciais para a compreensão das propriedades ópticas dos nanomateriais (KHAN, 2020). As nanopartículas são muito diminutas na nanoescala e seus elétrons não são livres como os seus materiais de origem. A consequência disso é que as nanopartículas reagem de forma diferente com a luz; quando a partícula se torna menor do que o comprimento de onda (KHAN, 2020). O fenômeno de SPR foi abordado no tópico 2.2.2.1 e, a seguir, uma breve explicação sobre confinamento quântico será exposta, bem como sua relação com os QDs.

Os QDs são nanomateriais semicondutores com menos de 100 nm de tamanho com éxcitons confinados nas três dimensões (DORFS *et al.*, 2011). Um éxciton ocorre quando o elétron da banda de valência migra para a banda de condução, deixando um buraco na mesma (Figura 9A). Quando o diâmetro ou tamanho do nanomaterial se aproxima ou se torna menor do que o raio de Bohr, os elétrons excitados tendem a formar uma ligação fraca com seu buraco (Figura 9B);

essa ligação entre elétron e buraco acontece via força de Coulomb e é chamada de éxciton; o raio de Bohr é a distância dentre o elétron e o buraco do éxciton (SUMANTH KUMAR; JAI KUMAR; MAHESH, 2018). Esse confinamento quântico é responsável pelas características únicas dos QDs como elevada absorção, larga região do espectro de excitação e estreitas bandas de emissão e fotoestabilidade (RAMALINGAM *et al.*, 2020).

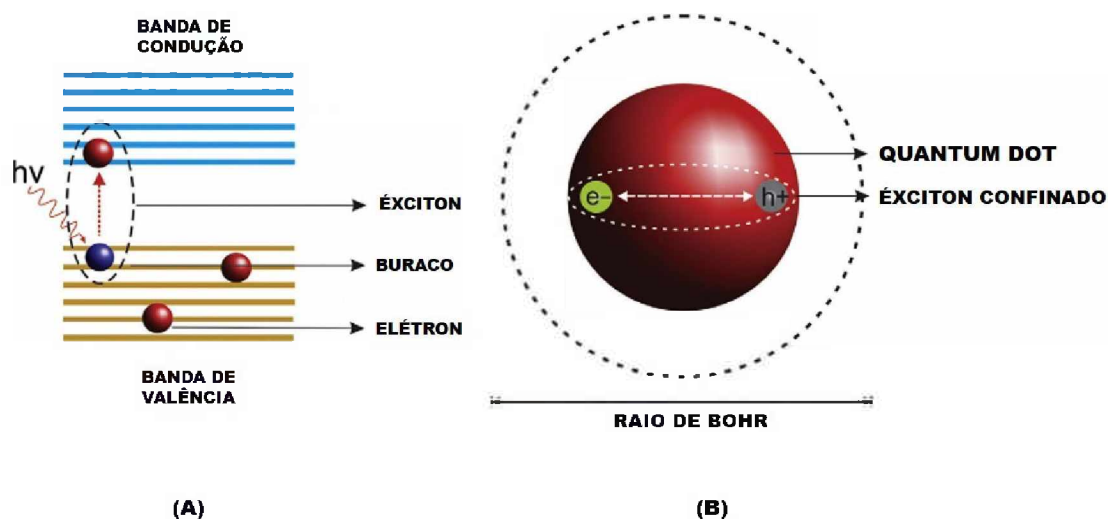


Figura 9. Formação de éxcitons em QDs (A) e comparação do tamanho de QDs e o raio de Bohr. Adaptado de Kumar, Kumar e Mahesh (2018).

Os Quantum Dots de Grafeno (GQDs) são nanomateriais luminescentes de dimensão zero da família do grafeno (CHEN *et al.*, 2017). Esses GQDs são fragmentos pequenos, na escala de 3 a 20 nm, e apresentam efeitos quânticos, além de extraordinárias propriedades ópticas e elétricas, bem como biocompatibilidade e baixo custo de produção (CHEN *et al.*, 2017).

O grafeno e seus derivados são extensamente explorados devido às suas propriedades eletrônicas. O grafeno é um nanomaterial 2D composto por folhas de carbono arranjados em forma de hexágonos com átomos de carbono ligados em sp^2 , podendo ser utilizado em sua forma pura ou derivados como óxido de grafeno (GO), óxido de grafeno reduzido (rGO) ou GQDs (SUARNAPHAET; PECHPRASARN, 2017).

As estratégias para o ancoramento de biomoléculas em biossensores baseados em grafeno são variadas, como é possível visualizar na Figura 10. O

grafeno fornece sítios ativos para interações com biomoléculas devido a sua área superficial. O GO e o rGO possibilitam a formação de epóxidos, hidroxilas e grupos carbonila na superfície e na lateral da folha de carbono (efeito de borda) e podem ser utilizadas para a interação com nanopartículas, QDs, DNA, enzimas, proteínas e outras moléculas específicas (SUARNAPHAET; PECHPRASARN, 2017). No entanto, o GO possui uma baixa condutividade e o grafeno puro é limitado quanto aos grupos funcionais. Os GQDs conseguem se sobressair quanto a essas desvantagens, uma vez que as pequenas folhas de grafeno dos GQDs fornecem mais sítios ativos, uma maior área superficial e mais extremidades acessíveis do que o grafeno (CAMPUZANO; YÁÑEZ-SEDEÑO; PINGARRÓN, 2019). Além disso, o confinamento quântico e o efeito de borda (lateral) fazem com que esses nanomateriais tenham um transporte rápido de elétrons e excelente condutividade, tornando-os materiais ideais para transdução de sinal (PIERRAT; GAUMET, 2020).

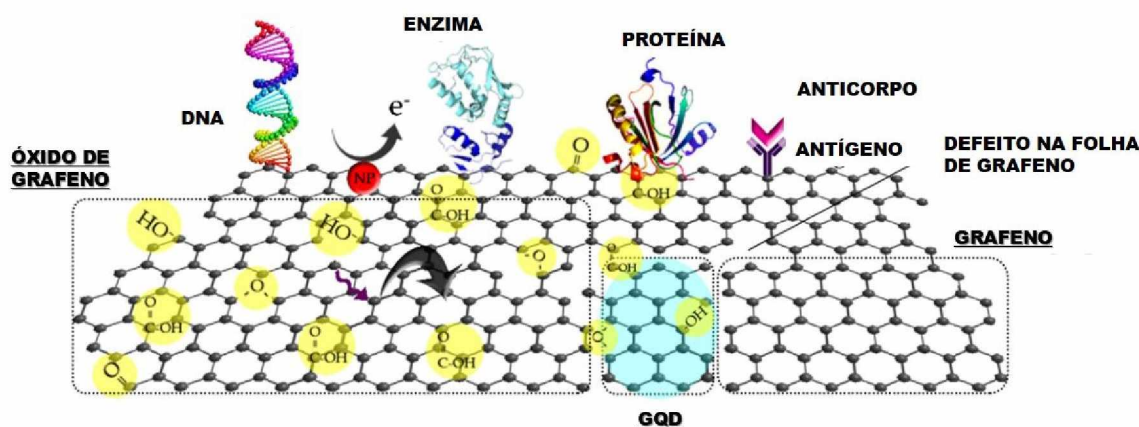


Figura 10. Ilustração esquemática dos materiais baseados em grafeno e biomoléculas que podem ser imobilizadas. Adaptado de Suvarnaphaet e Pechprasarn (2017).

Os nanomateriais podem ser sintetizados a partir de duas abordagens: *top down* ou *bottom up* (HADEF, 2018). Quando um material macroscópico é gradualmente processado até a formação de nanomateriais, tem-se a abordagem *top down*. Já quando há partículas nanométricas automontadas para formação de materiais, ocorre a abordagem *bottom up*. No caso dos GQDs, algumas abordagens *top down* são utilizadas, como técnicas hidrotermais, ablação química

e oxidação eletroquímica. Já os métodos *bottom up* são baseados na carbonização de precursores orgânicos por tratamento termal e possuem a vantagem de permitir um controle preciso da composição e propriedades físicas dos GQDs pela seleção dos precursores e condições para carbonização (CAMPUZANO; YÁÑEZ-SEDEÑO; PINGARRÓN, 2019).

As nanopartículas metálicas, como ouro e prata, têm se destacado e são extensamente exploradas devido às suas características, como as atividades catalíticas, biológicas, ópticas, eletroquímicas e eletrônicas (ZHANG; LIN, 2018). A ligação entre o analito e as AuNps pode alterar as propriedades físico-químicas, como a ressonância plasmônica de superfície, condutividade e comportamento redox; todas essas alterações ocasionam sinais detectáveis (PINGARRÓN; YÁÑEZ-SEDEÑO; GONZÁLEZ-CORTÉS, 2008).

As AuNps são facilmente conjugadas com biomoléculas para o reconhecimento de porções de anticorpos e oligonucleotídeos, por exemplo (KHAN *et al.*, 2022). Na aplicação em biossensores eletroquímicos, as AuNps permitem a transferência eletrônica mais eficiente e aumentam a área superficial, resultando em um aumento de área para a imobilização de biomoléculas (CAO *et al.*, 2021).

As AuNPs são sintetizadas por métodos *bottom up* ou *top down*, assim como os QDs de grafeno (KHAN *et al.*, 2022). No primeiro caso, as nanopartículas são preparadas pela agregação de átomos ou moléculas como nas técnicas de deposição química à vapor, síntese coloidal e por microemulsão. No segundo caso, o material de origem é reduzido por métodos físicos e químicos, como ablação a laser.

Neste trabalho, além dos nanomateriais, o 4-aminotiofenol (4-ATF), um composto orgânico com propriedades eletroquímicas e ópticas, tem grande aplicação na modificação de superfícies por camadas automontadas, principalmente em conjunto com ouro (ABACI; SHANNON, 2005; ÜZER *et al.*, 2014). A estrutura do 4-ATF lhe confere uma função de agente bifuncional que permite a interação com diversas moléculas e nanoestruturas, sendo utilizada no direcionamento de reações, principalmente pela ligação covalente entre o grupamento tiol e o ouro (ENI-OLORUNDA *et al.*, 2010; RISKIN *et al.*, 2008). Além

disso, a sua aplicação melhora a reprodutibilidade e estabilidade de plataformas eletroquímicas (BILLAH *et al.*, 2010; ÜZER *et al.*, 2014; YOLA *et al.*, 2013).

4. Biossensores eletroquímicos para detecção de HIV

Os biossensores eletroquímicos se destacam na área diagnóstica por várias razões já elucidadas ao longo do texto mas, principalmente, pela possibilidade de miniaturização e baixo custo de produção. Essas características vão de encontro com as propriedades ideais de um método diagnóstico, definidas, segundo Land *et al.* (2019) como as siglas atualizadas REASSURED; cada letra da palavra significa uma característica ideal como a conectividade em tempo real (R - *Real-time connectivity*), facilidade na coleta de amostras (E - *Ease of specimen collection*), acessibilidade (A - *Affordable*), sensibilidade (S - *Sensitive*), especificidade (S - *Specific*), interface amigável (U - *User-friendly*), rápido e robusto (R - *Rapid and robust*), livre de equipamentos (E - *Equipment free or simple*) e que chegue às pessoas que precisam (D - *Deliverable to end-users*) (LAND *et al.*, 2019).

Como discutido no item 1.3, vários biomarcadores virais podem ser detectados ao longo do curso da infecção por HIV, como o RNA, proteínas antigênicas, anticorpos e células. No entanto, as pesquisas na área de biossensoriamento de HIV estão focadas, atualmente, em tecnologias para áreas de recursos limitados e que consigam monitorar a carga viral (FARZIN *et al.*, 2020). Nesse sentido, a detecção de RNA viral se destaca, uma vez que é possível quantificar a carga viral e é padrão no monitoramento de terapias antivirais (SCHÜPBACH, 2003). Além disso, os genossensores que são utilizados para detecção de RNA ou DNA possuem vantagens em relação às outras formas de biorreconhecimento, como enzimas e anticorpos, já que possuem alta tolerância térmica e fácil modificação (WANG, 2000).

Nos últimos anos, vários genossensores eletroquímicos foram desenvolvidos para detecção de DNA específico para o HIV, principalmente por técnica de VPD (GONG; WANG; YANG, 2017; HU; LI; LI, 2018; LI *et al.*, 2020; LI *et al.*, 2014; LU *et al.*, 2021; SHAMSIPUR *et al.*, 2021). Li *et al.* (2020) desenvolveram uma plataforma para detecção do gene do HIV por VPD, usando óxido de grafeno reduzido com aminas automontadas (NH₂-rGO) e β -ciclodextrina

(β -CD) na superfície do eletrodo de carbono vítreo (ECG). O azul de metileno (AM) foi utilizado como indicador de hibridização e os autores chegaram em um limite de detecção de 8,7 fM. Outra estratégia utilizando AM foi desenvolvida por Lu *et al.* (2021); em um eletrodo flexível à base de papel foi construído um compósito baseado em estrutura metal-orgânica de níquel (Ni-MOF), nanopartículas de ouro, nanotubos de carbono e álcool polivinílico para detecção de DNA de HIV com um limite de detecção de 0,13 nM. Esses genossensores possuem boa sensibilidade mas não têm longa estabilidade.

Os GQDs são nanoestruturas que começaram a ser exploradas em 2008 e estão sendo muito estudadas nos últimos anos, principalmente em biossensores ópticos (GHAFFARKHAH *et al.*, 2022). Os genossensores eletroquímicos baseados em GQDs para HIV não são numerosos quando comparados aos ópticos, principalmente para detecção de RNA. Um trabalho recente envolveu o desenvolvimento de um genossensor com eletrodos impressos (SPE) modificados com quantum dots de óxido de grafeno (GoGQDs) para detecção de um microRNA (miRNA) específico para o câncer de próstata. Esses SPE foram conectados a dispositivos móveis e atingiu o limite de detecção de 0,091 pM (AKIN *et al.*, 2022). Outro trabalho visou o desenvolvimento de genossensores para detecção das bases nitrogenadas guanina e adenina; os eletrodos foram modificados com GQDs dopados com boro e carregados com ouro de forma inédita por meio de método ultrassônico (KAIMAL *et al.*, 2022).

Os biossensores que utilizam aptâmeros estão sendo mais utilizados para explorar as propriedades eletrônicas dos GQDs (MAHMOUDI-MOGHADDAM; TAJIK; BEITOLLAHI, 2019; ELSHAFFEY; ABO-SOBEHY; RADI, 2021; ZHU *et al.*, 2022; RUIYI *et al.*, 2022; CENTANE; NYOKONG, 2023). Aptâmeros são pequenas sequências de oligonucleotídeos altamente específicas selecionadas pela técnica SELEX (Systematic Evolution of Ligands by EXponential Enrichment) e são mencionados aqui como genossensores, uma vez que também são compostos de material genético.

O presente trabalho teve como objetivo o desenvolvimento de dois biossensores eletroquímicos para detecção de RNA de HIV. Eletrodos SP com tinta de carbono foram desenvolvidos e aplicados de uma maneira que o custo de

produção final seja baixo e o método de montagem seja fácil. Esse eletrodo foi modificado com monômero 4-ATF e AuNps no primeiro trabalho e com GQDs e AuNps no segundo.

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CAPÍTULO 2

Carbon ink-based electrodes modified with nanocomposite as a platform for electrochemical detection of HIV RNA

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Carbon ink-based electrodes modified with nanocomposite as a platform for electrochemical detection of HIV RNA

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Abstract

This work reports a new and easy-to-use methodology for construction of screen-printed electrodes (SPE) based on carbon ink and its modification with 4-aminothiophenol (4-ATP) and gold nanoparticles for detection of HIV RNA.

Morphological analysis for SPE/4-ATP/AuNps+DNA probe showed increased roughness indicating that 4-ATP monolayer works as a network for connection of functionalized gold nanoparticles with specific oligonucleotide probe to the RNA target. The biosensor response was monitored by indirect electrodetetection using ethidium bromide as indicator of duplex formation. The peak current response of ethidium bromide was linear related to the logarithm of concentration of HIV RNA, detecting up to 6 copies/mL and linear range of 6 to 11000 copies/mL of RNA, covering the clinically relevant concentration range (50-200 copies/mL). The biosensor discriminates negative and positive samples, making it possible to quantify the viral load, a crucial factor for the development of biosensors for the early detection of HIV.

Keywords: human immunodeficiency virus; screen-printed carbon electrode; electrochemical biosensor; HIV RNA viral detection; 4-aminothiophenol; gold nanoparticles.

Resumo

Este trabalho descreve uma nova metodologia e de fácil construção de eletrodos *screen printed* (SPE) baseados em tinta de carbono e sua modificação com nanopartículas de ouro e 4-aminotiofenol (4-ATF) para detecção de RNA do HIV.

A análise morfológica da sonda SPE/4-ATP/AuNps+DNA mostrou um valor de rugosidade aumentada, indicando que a monocamada de 4-ATF funciona como uma rede para conexão de nanopartículas de ouro funcionalizadas com sonda de oligonucleotídeos específicos ao alvo de RNA. A resposta do biossensor foi

monitorada por detecção indireta por voltametria de pulso diferencial utilizando brometo de etídio como indicador de formação duplex. O pico de resposta da corrente do brometo de etídio foi linear ao logaritmo de concentração de RNA do HIV, detectando até 6 cópias/mL e faixa linear de 6 a 11000 cópias/mL, cobrindo a faixa de concentração clinicamente relevante (50-200 cópias/mL). O biossensor discrimina amostras negativas e positivas, possibilitando quantificar a carga viral, um fator crucial para o desenvolvimento de biossensores para a detecção precoce do HIV.

Palavras-chave: vírus da imunodeficiência humana; eletrodo de carbono; biossensor eletroquímico; detecção do RNA de HIV; 4-aminotiofenol; nanopartículas de ouro.

1. Introduction

Human Immunodeficiency Virus (HIV) was initially reported in the early '80s, since then, it affects generations despite enormous advances in testing and treatment. According to the Joint United Nations Program on HIV/AIDS (UNAIDS), in 2019, 38 million people were affected by HIV [1]. HI is a lentivirus of the *Retroviridae* family, having in common the genome composed of RNA that replicates through reverse transcriptase (RT). Continuous replication leads to immunological impairment through the destruction of CD4 lymphocytes. The destruction of immune system cells causes the appearance of opportunistic infections, such as pneumonia and tuberculosis, defining the Acquired Immunodeficiency Syndrome (AIDS) [2]. The success of antiretroviral treatment is dependent on early detection and continuous monitoring of viral load in affected individuals [3]. HIV detection at the beginning of infection leads to early initiation of antiretroviral treatment, which improves life's quality of individuals living with HIV, since they help reduce viral load and consequently strengthen the immune system, a recurrent target of the disease [4].

The diagnosis of HIV is performed by serological and molecular methods; the first one aims to detect antibodies against HIV-1-2 and p24 antigens, namely Enzyme-Linked Immunosorbent Assay (ELISA) and Western Blot; the second

methodology detects nucleic acids mainly by amplification technology, such as Reverse Transcription Polymerase Chain Reaction (RT-PCR) [5]. Until 10 days from the onset of infection, called the “eclipse period”, there are still no commercially available serological and molecular tests. Nucleic acid-based methods can detect DNA or RNA at the end of the eclipse period and serological methods detect p24 protein after 14 days [6]. Twenty-one days after the onset of infection it is possible to use serological methods for detection of antibodies and this period, between the first day until 21 days, is called "acute infection" and the virus detection is only possible by nucleic acids and protein p24 detection [5]. However, molecular diagnostic methods present several disadvantages, such as the high cost of commercial kits, which is particularly harmful in countries with limited public health resources [7]. In addition, the high level of automation required, as well as the need for specialized people, constitute a barrier in the early, rapid and inexpensive HIV detection, interfering in the rapid start of antiretroviral therapy [8].

The term "ASSURED tests" was coined to represent an ideal diagnostic test for areas with limited resources for diagnosis of major epidemics, namely accessibility, sensitivity (low rate of false negatives), specificity (low rate of false positives), simple performance which requires little training, speed, robustness, free of equipment and able to reach those in need [9]. In this context, biosensors, along with microfluidics and nanotechnology, can meet these criteria, since they require small volume of samples, easy data interpretation and a rapid and early diagnosis to the ones in need [10].

Electrochemical biosensors stand out due to the possibility to develop low-cost microelectronic circuits and miniaturization [11]. The efficient joining of signal transduction and biorecognition components enables the development of several platform configurations for sensitive and selective detection of the target analyte, starting from the creation of new materials for biomolecules immobilization to new forms of electrochemical detection [12]. There are numerous components of HIV that can be detected through redox changes on the surface of an electrode. The components work as biomarkers in the detection of the disease, such as those directly linked to HIV, namely viral protein p24 [13] and viral RNA [14], as well as

components of the immune system, antibodies and TCD4 lymphocytes [15], for example.

Nucleic acids are biomolecules with desirable intrinsic characteristics to biorecognition processes in biosensors, since they present a sequence-specific hybridization behaviour with their complementary target [16]. The electrical properties of both single-strand DNA (ssDNA or RNA) and double-strand DNA (dsDNA) change before and after the hybridization process [17]. This change is detectable in terms of electron transfer by electrochemical techniques, such as cyclic voltammetry (CV), square wave voltammetry (SWV) and differential pulse voltammetry (DPV), by monitoring the oxidation and/or reduction of nucleic acids bases (label-free detection) or using labels to monitor the hybridization process [11].

The application of screen-printed carbon electrodes (SPCE) for biosensing is widely studied [18, 19]. They consist of high portability and market-friendly devices, being applied to a low cost and facile production on a wide scale, for example, the commercially well-established glucose biosensor [20]. Analytical parameters such as sensitivity and selectivity of SPCE-based platforms can be increased with the use of materials like conductive polymers and nanomaterials [18]. In addition, important factors to overcome barriers between the applicability of scientific development and commercial use, namely stability and reproducibility can be achieved through the association between electrochemical and nanomaterial biosensors [21, 22].

The 4-aminothiophenol (4ATP) is an organic molecule with electrochemical and optical applications due to its characteristics such as the ability to form self-assembled layers (SAMs), forming nanomaterial networks and it works as a bifunctional agent [23]. The thiol group of its structure makes it a versatile material, mainly in methodologies involving gold electrodes and gold nanoparticles (AuNps) [24]. AuNps have a high surface-to-volume ratio and high surface energy to provide a stable immobilization of a large amount of biomolecules and retention of their bioactivity, also reflecting on better results in stability, reproducibility and sensitivity [22].

Herein, due to the lack of accessible ASSURED HIV diagnosis, this work reports a facile methodology to construct screen-printed carbon electrode (SPCE)

in a manufactured way and its application in a novel platform using SPCE modified with 4-ATP and AuNps conjugated with a specific DNA capture probe to offer a low cost, selective and sensitive alternative for electrochemical biosensing to detect the genomic RNA of HIV in human plasma.

2. Material and methods

2.1 Reagents and materials

Sulfuric acid (99%), gold (III) chloride trihydrate ($\geq 99.9\%$), graphite flakes ($> 99.9\%$) potassium ferricyanide ($\geq 99.5\%$), hexaammineruthenium (II) chloride $\text{Ru}(\text{NH}_3)_6\text{Cl}_2$ (98%), 4-aminothiophenol (97%), ethidium bromide ($\geq 99\%$) were purchased from Sigma-Aldrich. Disodium hydrogen phosphate ($\geq 99\%$), monosodium hydrogen phosphate ($\geq 98\%$), sodium citrate dihydrate and potassium chloride ($\geq 99\%$) were purchased from Neon. Butylglycol acetate ($> 98\%$), vinyl resin (A.P), carbon black, silver nitrate (A.P), potassium hexacyanoferrate (III) $\text{K}_3[\text{Fe}(\text{CN})_6]$ ($>99\%$), potassium hexacyanoferrate (II) $\text{K}_4[\text{Fe}(\text{CN})_6]$ ($> 99.5\%$), methyl isobutyl ketone (99%) were purchased from Dinâmica, Mexichem S/A, Arinos, Qhemis, Fluka Analytical and Acros Organics, respectively.

The design for the printed electrode was created in Corel Draw 2018® and the molds were printed with a Roland® 45° needle on the vinyl adhesive with dimensions of 210 mm by 297 mm (dimensions of an A4 sheet). These dimensions cover the space needed to print 63 templates electrodes. The printed substrate for the manufactured SPCE was polyvinyl chloride (PVC). Two vinyl adhesive templates were used in the construction of the SPCE. The first one contains the template design of the working, counter, reference electrodes and the wires/tracks that connect them to the electrochemical device. The second adhesive is placed at the end of the print on top of the electrode and demarcates the electroanalytical area.

2.2 Apparatus

The SPCE has been connected to the electrochemical workstation via DropSens screen printed electrode adapter. All electrochemical techniques of this work were performed in Autolab potentiostat (Model PGSTAT302N, Metrohm) with NOVA software. The surface morphology of the electrodes were assessed by scanning probe microscopy (Model 5100 N, Hitachi), performed in the dynamic force microscope (DFM) mode, and by scanning electron microscopy (SEM) on an EVO MA10 Zeiss microscope.

2.3 Preparation of carbon and silver inks

Carbon conductive ink was prepared to be printed as a working electrode and counter electrode (Figure 1A). The silver ink was printed as a reference electrode (Ag^0). Silver ink was prepared using two solutions, one with 10g of silver flake, produced according to Paula *et al.*, [25], in 1.9 mL of butylglycol acetate and another composed of 10 mL of vinyl resin 20% in methyl isobutyl ketone. The second solution was heated to 50°C under agitation until its complete solubilization. Once solubilized, 1.022g of this solution was added to the first prepared solution containing silver flakes. The mixture was kept in constant agitation and heating until its homogenization.

For the carbon ink, first, 1.6g of vinyl were solubilized in 11 mL of methyl isobutyl ketone with heating at 50°C under constant agitation. After complete solubilization, 7.3g of graphite flakes (325 mesh), 1.6g of carbon black, and 9.0 mL of butylglycol acetate were added. Then, agitation was maintained at 50°C until the ink was completely homogenized. Both silver and carbon ink were stored at 8° C.

2.4 SPCE/ Ag^0 construction

The first step for SPCE/ Ag^0 construction is glueing the first vinyl adhesive on PVC of 5.0 mm thickness. This vinyl adhesive contains the template of the electrode track and where the paints will be printed (Fig. 1a). Thus, the adhesive is removed and exposes the path of each electrode. Both inks are printed with a rectilinear spatula and kept at 55°C for 25 minutes for complete drying (Fig. 1b). Finally, the first adhesive template is entirely removed and another one is placed on

top of the electrode (Fig. 1c), then, only the working, reference and counter electrodes are exposed for future experiments (Fig. 1d).

a

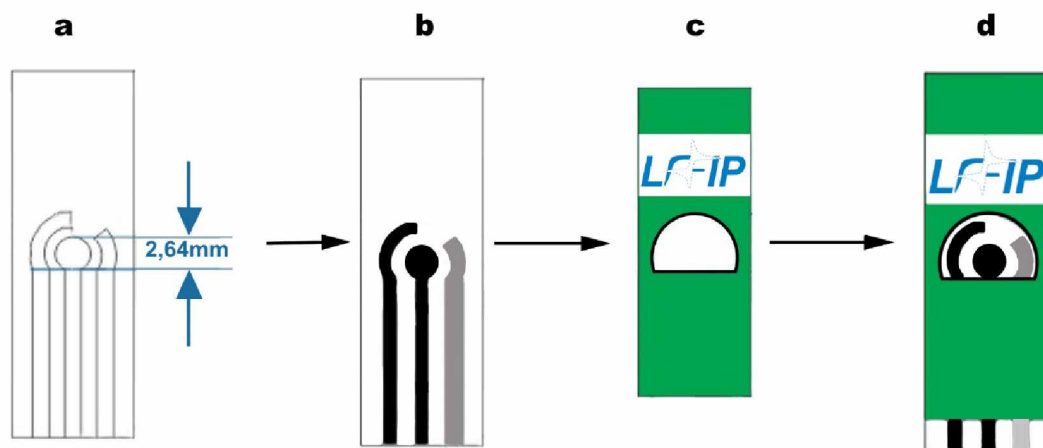


Fig. 1. SPCE/Ag⁰ construction. **(a)** First template vinyl adhesive with working, counter and reference electrodes tracks. **(b)** After removing the adhesive tracks, carbon and silver inks are printed with a spatula. **(c)** Second template adhesive is used to isolate and delimit the electroanalytical area. **(d)** Final SPCE/Ag⁰ construction.

2.5 Gold nanoparticle synthesis

The AuNps synthesis method was based on the Turkevich-Frens methodology [26, 27]. Basically, in this method, sodium citrate is used to reduce gold salts, which leads to the nucleation and growth of gold nanoparticles with a size of 15 to 20 nm, stabilized by citrate ions. Subsequent to the synthesis, the AuNps were stored at 4 °C until their use. The absorption spectrum was analyzed in a UV-VIS spectrophotometer in quartz cuvettes (1 cm optical path). A 1:1 dilution of AuNps in deionized water was prepared and the spectrum was analyzed at 300 to 700 nm to observe the peak absorption at 520 nm, wavelength that refers to gold nanoparticles with sizes between 15 and 20 nm.

2.6 SPCE/4-ATP/AuNps-HIV1S platform

The electrodes produced (Fig. 2a) undergo electrochemical polishing through the CV technique and the electrodes with reproducible electrochemical profiles are selected for the later stages. Electropolishing is performed in 0.5M sulfuric acid solution and phosphate buffer (0.1 mol/L, pH 7.4) between 0.00 V to +1.00 V, 20 cycles at 500 and 100 mV/s.

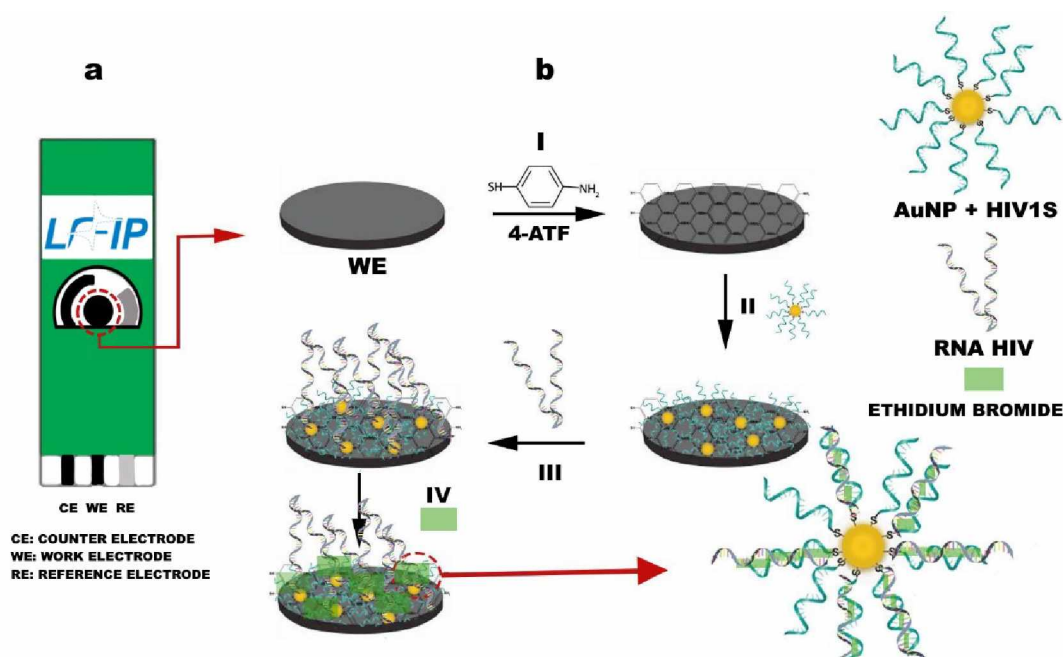


Fig. 2. Schematic illustration of SPCE/ATP/AuNps-HIV1S platform construction. **(a)** SPCE/Ag⁰. **(b)** Sequence of the modifications on the working electrode surface. I: monomeric modification with 4-aminothiophenol. II: addition of the previously functionalized AuNps with the thiolate capture probe HIV1S. III: Plasma-enriched HIV viral RNA. IV: DNA intercalant used as an indicator of the hybridization reaction.

The thiolate oligonucleotide sequence HIV1S (5' - **SHAATTGGAGAGCAATGGCTAGTGA**-3') used in this work [28] as the probe was purchased from Invitrogen and prepared in a phosphate buffer (0.1 mol/L, pH 7.4). The evaluation of the platform's selectivity was carried out using Zika virus genomic RNA (gRNA) and *Staphylococcus aureus* bacteria genomic DNA (gDNA) as negative control under the same conditions as the positive HIV gRNA (positive

target). All the gRNA samples were purified from plasma samples using an ABBOTT m2000sp extractor and quantified by RT-PCR at the Adolf Lutz Institute (SP) and by spectrophotometer (BioDrop®) prior to electrochemical detection. The use of human samples in this work is regulated by the Human Research Ethics Committee by the registration number 4348672.

The HIV-specific probe (HIV1S) contains a thiol group at the 5' end of the oligonucleotide sequence for interaction between this group and AuNps. Thus, 2 μ L of HIV1S (10 μ g/mL, phosphate buffer 0.1 mol/L, pH 7.4) in 200 μ L of AuNps were added to occur the functionalization of AuNps with the probe. This interaction occurs at room temperature for 1h.

For surface modification with the 4-ATP monomer, 5 μ L of 5 mM in sulfuric acid 0.5 M was dripped on the electrode (Fig. 2b - I) and left for 1h at room temperature. Subsequently, the electrode was washed in deionized water for 6 seconds. Then, the DPV was performed in phosphate buffer with modulation 0.07 V, pulse interval 0.20 s and velocity of 0.03 V/s between + 0.20 and + 0.90 V. This potential window covers the oxidation of EB [(3,8-diamino-5-ethyl-6-phenyl phenatridinium bromide)] that was used as an indicator of hybridization reaction between the probe (HIV1S) and its target, the complement in the RNA molecule of the HIV virus (Fig. 2b - IV). Thus, the baseline resulting from this step is extracted from the final result after interaction of the EB with the target probe.

After surface modification with 4-ATP monomer, 5 μ L of the AuNps-HIV1S probe (Fig. 2b - II) were added in the electrode and left at room temperature for 30 minutes. After this period, the electrode was washed with phosphate buffer for 6 seconds.

In parallel, CV in the presence of anionic redox probe $\{K_3[Fe(CN)_6]/K_4[Fe(CN)_6]\}$ and cationic probe $\{[Ru(NH_3)_6]Cl_2\}$ (5.0 mmol/L) containing KCl (0.10 mol/L), $v = 50$ mV/s was performed to analyze the properties of electronic transfer in the electrode-solution interface comparing bare SPCE, SPCE with the 4-ATP monomer and SPCE with 4-ATP and AuNps.

Peak data were corrected by subtracting a straight line tangent to the base, adapting vertically and their characteristics (area and height) were calculated by the peak analyzer tool, included in the Origin 8 software. The peak current (I_p) was

obtained by the necessary height in the graphs of current per electric potential and the electrochemical charge (Q) was obtained from the area under the curve integrating the area under the curve of the voltammograms, based on the graphs of current per time.

2.7 Electrochemical detection of HIV RNA

After the bioelectrode construction (SPCE/4-ATP/AuNps-HIV1S), gRNA of the HIV virus was detected. All viral RNA samples from infected patients were enriched with diluted plasma from healthy patientes (1:10, V/V) in phosphate buffer (0.1 mol/L, pH 7.4) in order to mimetize the natural environment in which viral RNA is found in the body. 5 μ L of enriched plasma were dripped on the electrode and left at 55°C for 20 minutes. After, the electrode is washed in phosphate buffer for 6 seconds to remove biomolecules that have not interacted with the probe. Ethidium bromide (EB) at 0.1 mg/mL, 5 μ L were added in the electrode for 10 min at room temperature and then washed in deionized water for 6 seconds. DPV was performed in phosphate buffer with the same parameters described in item 2.6.

2.8 Figures of merits of biosensor

Performance of the genosensor was evaluated by variation of the concentrations of HIV gRNA plasma-enriched (1:10, V/V). Biosensor calibration curve was constructed with gRNA concentrations of 6, 56, 560, 1100 and 11000 copies/mL (Cp/mL), diluted 1:10000, 1:1000, 1:100, 1:50 and 1:5 in plasma, respectively.

Biosensor stability was evaluated after storage at 8 °C for 50 days using bioelectrodes (SPCE/4-ATP/AuNps-HIV1S) stored hermetically sealed with nitrogen gas (N₂) with (wet-chamber) and without (dry-chamber) humidity in the environment.

3. Results and discussion

3.1 Characterization of the SPCE platform

In the presence of the anionic redox pair (Fig. 3a) there was an increase in the anodic and cathodic peak currents (I_{pa} and I_{pc} , respectively) in SPCE, SPCE/4-ATP, SPCE/4-ATP/AuNps, respectively. Compared to bare SPCE, I_{pc} and I_{pa} increase 2.8x and 3.5x in SPCE/4-ATP, SPCE/4-ATP/AuNps, respectively. When analyzing the electrical charge (Fig. 3a inset), there is an increase of 2.14x and 2.76x in SPCE/4-ATP and SPCE/4-ATP/AuNps, compared to bare electrode. Therefore, it is observed that the progressive change in surface modifications occur in order to facilitate the electronic transfer of the redox pair, suggesting that the 4-ATP film have a positive net charge, thus attracting the ferrocyanide ions, since the predicted pK_a of 4-ATP is 8.74, conferring a positive charge in the worked 7.14 pH. Corroborating the findings regarding the facilitation in electronic transfer, the results of electrochemical impedance spectroscopy (EIE), presented in Fig S.1 and Fig S.2, show that the resistance to charge transfer value (R_{CT}) decrease 1.89x and 2.22x when the bare SPCE (1.51 ± 0.03 k Ω) is compared with the SPCE/4-ATP (0.80 ± 0.05 k Ω) and the SPCE/4-ATP/AuNps (0.67 ± 0.05 k Ω), respectively (Supplementary Material).

A reverse behavior was observed in the presence of the cationic probe (Fig. 3b), where the 4-ATP modification decreases the peak current, reinforcing that 4-ATP forms a positively charged layer and repels the positively charged $[Ru(NH_3)_6]^{+2}$ ion. However, Balvedi *et al.* [29], evaluated the behavior of the electrochemical polymerization of the 4-ATP monomer and obtained opposite results to this study, showing that poly(4-ATP) presents anionic behavior comparing interactions with anionic $\{K_3[Fe(CN)_6]/K_4[Fe(CN)_6]\}$ and cationic $\{[Ru(NH_3)_6]Cl_2\}$ probes. This difference is expected since monomeric oxidation and polymeric elongation alters electrostatic interactions between the solution's monomer and in this present work the monolayer rearranges spontaneously in the carbon electrode.

The bare carbon ink working electrode showed a roughness coefficient (R_q) of 138.6 ± 9.2 nm, Fig. 3c - I, and it was possible to see in the SEM images the uncovered carbon flakes of the ink (Fig. 3c - IV). When the electrode was modified with 4-ATP, there was a decrease in the roughness value ($R_q = 112.1 \pm 7.5$ nm, Fig. 3c - II), probably due to the deposition of the monomer in the surface valleys, and the SEM images showed a more homogenous surface (Fig. 3c - V). After the

addition of the AuNPs modified with the DNA probe there was an increase in the roughness value ($R_q = 147.1 \pm 9.5$ nm, Fig. 3c - III) and it was possible to observe punctual structures peaks with average size of 200 nm, which can be related to the fact that the nanoparticles were adsorbed on the electrode after the drying step. Meanwhile, the SEM images (Fig. 3c - VI) showed a more heterogenous surface in comparison with the carbon ink electrode.

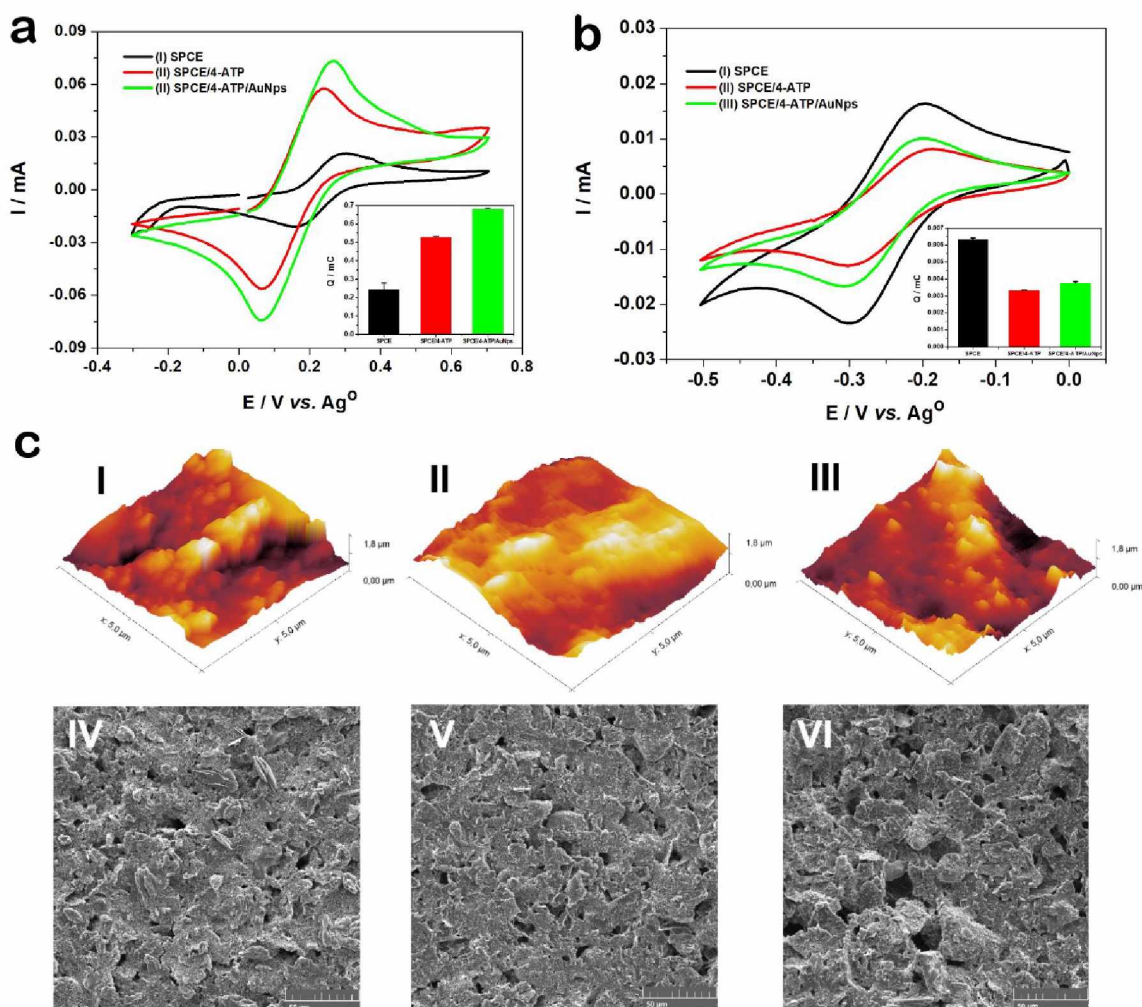


Fig. 3. (a) Cyclic voltammograms with anionic probe: bare SPCE (I), SPCE/4-ATP (II) electrode, and SPCE/4-ATP/AuNps electrode (III). Inset: histograms of the electrical charge involved in the oxidative and reductive processes. Electrolyte: $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (5.0 mmol/L) solution containing KCl (0.10 mol/L), $\nu = 50$ mV/s. (b) Cyclic voltammograms with cationic probe: bare SPCE (I), SPCE/4-ATP electrode (II), and SPCE/4-ATP/AuNps electrode (III). Inset: histograms of the electrical charge involved in the oxidative and reductive processes. Electrolyte:

[Ru(NH₃)₆]Cl₂ (5.0 mmol/L) solution containing KCl (0.10 mol/L), $\nu = 50$ mV/s. **(c)** SPM and SEM images of the bare SPCE (I, IV), SPCE/4-ATP (II, V) and WE/4-ATP/AuNps+DNA Probe (III, VI).

3.2 Functionalization and electrochemical detection of HIV genomic RNA

Ethidium bromide (EB) is a planar aromatic organic compound capable of interacting with the nitrogenous bases of nucleic acids [30]. Detection is possible since EB is electroactive and exhibits different electrochemical behaviors with single-strand and double-strand DNA. Previous studies demonstrated the effect of EB accumulation on the electrode surface when probe and target hybridization occur due to DNA duplex formation, caused by interaction with paired bases. On the other hand, when hybridization does not occur, there is no large accumulation of EB on the electrode surface and the current response is lower [31]. This mechanism is observed using the present bioelectrode by differential pulse voltammetry, shown in Fig. 4a. The BE presented electroactivity on the SPCE/4-ATP/AuNps-HIV1S platform with peak oxidation at approximately + 0.58 V vs. Ag⁰.

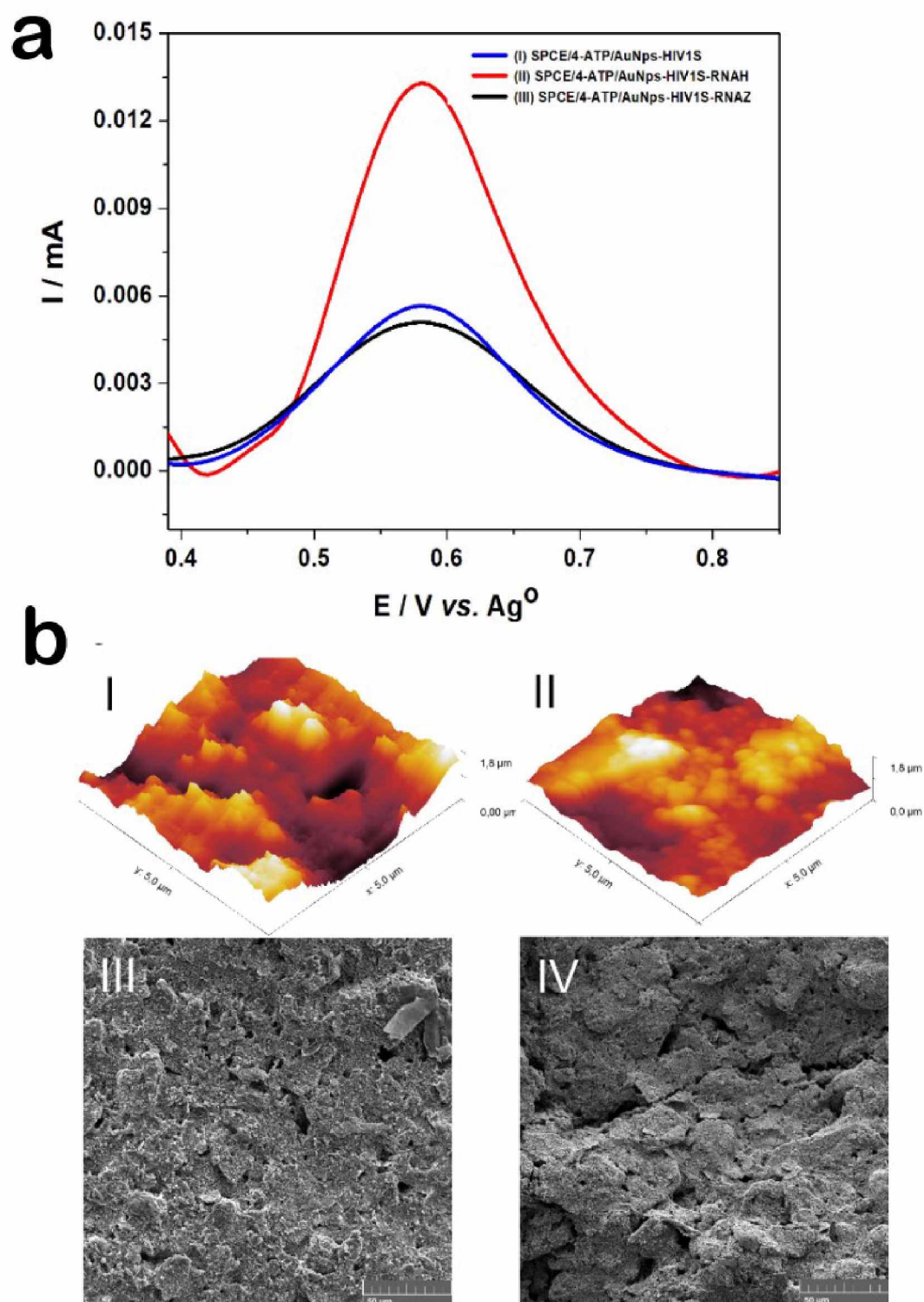


Fig. 4. (a) Differential pulse voltammograms of screen-printed carbon electrode (SPCE) modified with 4-ATP containing AuNps-HIV1S in the presence of ethidium bromide before (I) and after interaction with the complementary target (HIV-RNA, II)

and non-complementary target (HIV-RNA, II) and non-complementary target (ZIKA-RNA, III). Electrolyte: phosphate buffer (0.10 mol/L), pH 7.4, amplitude 0.07 V, pulse interval, 0.2 s, $v = 30$ mV/s. **(b)** SPM and SEM images of WE/4-ATP/AuNps+DNA Probe/ZIKA RNA (I, III) and WE4-/4-ATP/AuNps+DNA Probe/HIV DNA (II, IV).

The current response change is observed when the complementary and non-complementary targets interact with the modified electrode (Fig. 4a). The bare platform with the AuNps-HIV1S probe (Fig. 4a - I) shows the current peak at 4 ± 0.13 μ A and when the HIV viral RNA complementary target (Fig. 4a - II) is added, the current increases by 3.5 times (14 ± 0.3 μ A) and the current response of the non-complementary target of Zika virus viral RNA (Fig. 4a - III) presents the peak current near the probe response at 3 ± 0.2 μ A.

AuNps offer many sites of adsorption for biomolecules, mainly with the use of thiolate probes due to the strong interaction between gold and thiol groups [32]. The large surface area of the AuNPs also provides an efficient immobilization of biomolecules, which facilitates the detection of the target analytes [22]. The EB is a hydrophobic molecule with affinity for DNA-RNA double-strands, composed of its hydrophobic nitrogen bases in the hydrophilic aqueous environment of plasma, but not by single-strand of DNA or RNA [30]. The smallest signal of electrical current of the probe and the non-complementary target is expected in relation to the complementary target, resulting from the intercalation of EB to the double-strands formed. Thus, the platform SPCE/4-ATP/AuNps-HIV1S proved to be selective in the discrimination between positive and negative target in a complex sample of human plasma.

Scanning probe microscopy can be a valuable technique to evaluate the immobilization and hybridization of DNA and RNA in solid surfaces [33]. The addition of the negative RNA target (ZIKA RNA) did not significantly alter the roughness coefficient ($R_q = 139.3 \pm 10.2$ nm, Fig. 4b - I) in relation with the probe (Fig. 3c - III). However, the addition of the positive target (HIV RNA) increased the roughness coefficient ($R_q = 201.6 \pm 15.9$ nm, Fig. 4b - II) and it was possible to identify globular structures with average size of 500 nm. Double stranded nucleic acids are less flexible than single stranded nucleic acids [34], thus the hybridization

of the DNA oligonucleotide present on the electrode surface with the complementary RNA present in the sample creates more prominent clusters on the electrode surface [35]. It was also possible to observe different morphologies in the SEM images, with the addition of the positive target (Fig. 4b - IV) caused a change in the surface of the working electrode, making it more heterogeneous in comparison with the negative target (Fig. 4b - III). This may be due aggregation of the nanoparticles after adding the positive target.

Analytical responses by differential pulse voltammetry with different concentrations of HIV gRNA are shown in Fig. 5a *versus* the variation of the gRNA concentration logarithm in copies per mL (Cp/mL). Fig. 5b shows the current response *versus* the potential oxidation window of ethidium bromide. The order is from lowest concentration to highest concentration of the x axis: 6 ($5.33 \pm 0.40 \mu A$), 56 ($7.85 \pm 0.63 \mu A$), 560 ($11.00 \pm 0.60 \mu A$), 1100 ($12.00 \pm 0.46 \mu A$) and 11000 ($14.60 \pm 0.29 \mu A$) Cp/mL, diluted 1:10000, 1:1000, 1:100, 1:50 and 1:5 in plasma, respectively. The calibration curve of the genosensor was constructed, as the oxidation peak current of the EB indicator correlates with the logarithm of the concentration of genomic RNA of HIV. The experimental calibration curve can be fitted by a linear straight line of equation $I_p (\mu A) = 2.91 (\mu A/Cp/mL) \cdot \log[gRNA] (Cp/mL) + 3.04 (\mu A)$, $R^2 = 0.9989$ ($N = 3$). There was a linear response range between 6 Cp/mL (6.5 ng/mL) to 11000 Cp/mL (13 $\mu g/mL$). The biosensor detected up to 6 Cp/mL. This value is much lower when compared to commercial RT-PCR-based that detect up to 20, 30 and 40 Cp/mL [36].

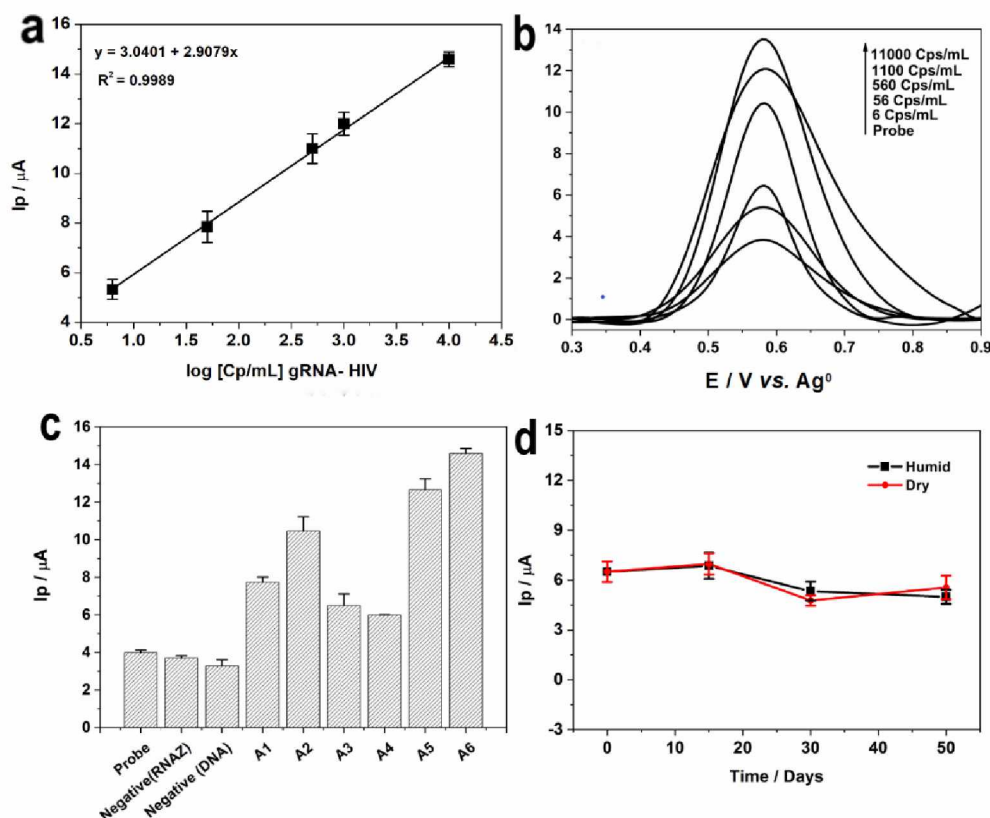


Fig. 5. (a) Calibration curve of the biosensor. **(b)** Differential pulse voltammograms in phosphate buffer. **(c)** Histogram showing the peak current (I_p) for positive: gRNA HIV-affected individuals (A1-A6) and negative samples of Zika virus RNA (RNAZ) and *Staphyloaureus* (DNA). **(d)** Storage stability in a humid and dry inert environment. Electrolyte: phosphate buffer (0.10 mol/L), pH 7.4. Modulation amplitude 70 mV, pulse interval 0.2 s; $v = 30 \text{ mV/s}$; $N = 3$.

Plasma samples dopped with HIV genomic RNA (length of ~10 kb) [37] were used to validate the biosensor. Figure 5C shows the response of 6 independent samples compared to the probe and two negative samples, one of Zika virus genomic RNA and another of genomic DNA of *S. aureus* bacteria. All samples differed from the probe and negative, showing the specificity and selectivity of the HIV1S probe with different genetic materials in a complex protein matrix, an intrinsic characteristic of human plasma. The recently developed platforms (Table 1) exhibit remarkable analytical parameters in biosensing, however, most use synthetic sequences of complementary DNA to validate the biosensor and not the complex

biomolecule of HIV RNA. This can be a major challenge with regard to the possibility of transforming technology at the level of research for commercial applications. This work uses the native RNA molecule in its environment, allowing it to be in its usual conformation. In addition, it is important to note that, as mentioned in item 2.6, different electrodes with the same electrochemical profiles were selected for all studies of this work. This demonstrates that our artisanal electrodes have high reproducibility for future commercial applications.

The concentrations of the positive samples were determined using the calibration curve data (Fig. 5c): (A1) 41.67 Cp/mL; (A2) 354.81 50 Cp/mL; (A3) 15.49 Cp/mL; (A4) 10.42 Cp/mL; (A5) 2000 Cp/mL; (A6) 10232 Cp/mL. The current varying linearly with the viral load indicates that in addition to the qualitative possibility of detection, it is also possible the quantitative detection of the viral load using this methodology in this response range. This is a crucial factor for the development of biosensors for the early detection of HIV, since it is only possible to detect the genetic material in the first days of infection and in very low concentrations. Thus, these results indicate an important step towards the development of quantitative and qualitative point-of-care diagnostics.

Recent electrochemical biosensors (Table 1) share good performance regarding the detection limit but have gaps in the detection of real viral RNA, since most detect synthetic DNA molecules. This work shows the possibility of detecting HIV viral RNA in a stable, sensitive and accessible manner. It is important to highlight that the present biosensor detects the range considered clinically relevant, between 50-200 Cp/mL, because it is related to resistance to antiretroviral therapy and is the focus of continuous monitoring in HIV-infected patients [38].

Table 1. Recent examples of electrochemical biosensor for HIV detection.

Electrode	Platform	Technique	Detection until	DLR	Stability	Sample Volume	Sample	Target	Reference
Commercial SPCE	Graphene-Nafion	CV/DPV	5 nM	40 nM to 2.56 μ M	30 days	50 μ L	Serum	Synthetic DNA oligo	[39]
GCE	Graphene-Nafion	EIS	2.3×10^{-14} M	1.0×10^{-13} to 1.0×10^{-10} M	1 week	5 μ L	Buffer	Synthetic DNA oligo	[40]
GCE	Molecularly imprinted polymers/MWCNTs	DPV	0.083 pg cm^{-3}	1.0×10^{-4} to 2 ng cm^{-3}	5 days	5 μ L	Serum	Protein	[41]
CPE	Ion-imprinted polymer nanoparticles/AuNps	DPV	0.3 fM	1 fM to 0.3 fM	Not reported	5 μ L	Serum	Synthetic DNA oligo	[42]
Au electrode	-	DPV	0.21 pM	1pM to 100nM	Not reported	100 μ L	Serum	Synthetic DNA oligo	[43]
GCE	Amino-reduced graphene oxide/ β -cyclodextrin	DPV	8.7 fM	0.05 pM to 1 pM	1 week	5 μ L	Serum	Synthetic DNA oligo	[44]
Artisinal SPCE	4-aminothiophenol/AuNps	DPV	6 Cp/mL (6.5 ng)	6 to 11000 Cp/mL (6.5 ng to 13 μ g)	15-30 days	3-5 μ l	Plasma	Viral Genomic RNA	This work.

DLR: Detection Linear Range; GCE: Glassy Carbon Electrode; MWCNTs: Multi-Walled Carbon Nanotubes; CPE: Carbon Paste Electrode;

Stability is an important parameter for future commercial applications of new biosensor platforms [45]. Carbon-based materials are inert due to the hydrophobic nature of the carbon network and disperse polar solvents poorly [46]. The functionalization of these platforms can influence both the change in the reactivity of carbon ink and the conformation of biomolecules inserted for biorecognition.

In the present work, to analyze the humidity factor in improving or worsening stability, the electrodes were stored in an inert atmosphere (N_2) with or without humidity in the environment (Fig. 5d). All the ready-to-use genosensors (SPCE/4-ATP/AuNps-HIV1S) were stored under the same conditions. For each evaluated day, DPV was performed to detect HIV RNA (target) in triplicate. The same target sample was used both on day 1 and on the rest of the days for comparison. The electrodes stored in a wet or dry chamber presented the same original response after 15 days. The current response maintained in a humid environment, on thirtieth and fiftieth day, the response dropped by 81.5% and b, respectively, and 73.5% and 85.5%, in the electrodes without humidity, respectively. Therefore, it is reasonable to infer that humidity influences the platform response up to 15 days of storage. Then, the response drops progressively in the humid environment and within 50 days there was a response's increase in the dry environment, probably due to operational problems, such as errors in pipetting and storage. The use of wet chamber has already been reported in previous work [47]. Hence, the use of dry storage helps in the reduction of biosensor's production stages. According to Table 1, our artisanal electrode is promising since stability is better or similar to new platforms for detecting HIV genetic materials using commercial electrodes. The reproducibility of our artisanal electrodes is 90%, that is, for every 10 electrodes manufactured, 9 have the same electrochemical profile.

4. Conclusions

Electrochemical biosensors are rapid, cheap and portable alternatives to conventional methods for detecting HIV biomarkers. There are also gaps in HIV diagnosis in the initial period of the disease, called acute infection stage. At this stage it is only possible to detect genetic materials of the virus and the p24 antigen and in very low amounts that, consequently, only sensitive methods achieve.

Here we present a new methodology for SPCE construction in a simple way, without many stages for production, using carbon ink and vinyl adhesives. The simplicity of the electrodes construction for biosensing is an important factor for future commercial applications, especially in areas with limited resources for HIV diagnosis.

The SPCEs were modified with a 4-ATP monolayer which improved the electronic properties of the carbon-based platform, besides constituting an anchoring network for gold nanoparticles functionalized with thiolate probe. The cationic characteristic of the monolayer was observed with its characterization using anionic redox probe $\{K_3[Fe(CN)_6]/K_4[Fe(CN)_6]\}$ and cationic probe $\{[Ru(NH_3)_6]Cl_2\}$. In addition, studies with SEM and SPM showed the roughness change with the use of 4-ATP and AuNps decorated with a thiolate probe specific to HIV.

EB is an efficient DNA intercalant and indicates the occurrence or not of hybridization due to its ability to bind to double-strand, but not to the single-strand of DNA. EB electroactivity was observed in +0.58V and the current response decreases when comparing the presence or absence of HIV RNA. The SPCE/4-ATP/AuNps-HIV1S bioelectrode was validated with real viral RNA samples enriched with blood plasma and non-complementary targets of RNA (Zika virus) and DNA (*S. aureus*), showing specificity and selectivity. The calibration curve was constructed and the current response of EB was linear related to the logarithm of concentration of HIV RNA, detected up to 6 Cp/mL and linear range between 6 and 11000 copies of RNA, covering the clinically relevant concentration range (50-200 Cp/mL) for monitoring antiretroviral treatment, detecting extremely low concentrations of the virus, an acute phase infection's characteristic. The stability of the genosensor was analysed in a dry and humid environment and it remained stable for 15 days in both conditions.

Furthermore, this work shows the possibility of simple construction of electrodes with commercial applications in point-of-care diagnosis for the detection of viral RNA, presenting sensitivity, selectivity, specificity and stability.

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CAPÍTULO 3

High performance label-free genosensor based on a carbon screen-printed electrode modified with graphene quantum dots and gold nanoparticles for HIV RNA detection

High performance label-free genosensor based on a carbon screen-printed electrode modified with graphene quantum dots and gold nanoparticles for HIV RNA detection

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Highlights

- Label-free electrochemical genosensor for HIV RNA detection was manufactured.
- Figure of merit was used to characterize the performance of the electrochemical device.
- Detection limit was 1 fg/mL and stability of 60 days.
- Validation was performed with plasma of HIV + and HIV - individuals.

Abstract

Electrochemical biosensors using nanomaterials shown a privileged possibility for the advancement of the analytical devices with potential for diagnostics due to the improves conductivity and optimizes the relationship area/volume. This work presents the construction of an HIV-specific genosensor through the modification of CSPE with graphene quantum dots decorated with L-cysteine and gold nanoparticles (cys-GQDs/AuNps). Cys-GQDs were characterized by FT-IR and UV-vis spectra and electronic characteristics were evaluated by cyclic voltammetry and impedance spectroscopy. Cys-GQDs showed a synergistic effect with AuNPs, improving the electronic transfer of the anionic redox probe $[\text{Fe}(\text{CN})_6]^{3-/4-}$ on the electrode surface and a increase of the electroactive area of 37 times when compared to the bare surface. Detection was performed label-free by differential pulse voltammetry monitoring the current response of the anionic redox probe. The genosensor was validated with real RNA samples and a calibration curve was constructed with a detection limit of 1 fg/mL (1 copy/mL). This new genosensor presents sensitivity, specificity, selectivity and stability in early HIV detection and the cys-GQDs and AuNps-based platform improves overall genosensor performance, which are important features for the application of biosensors commercially.

Keywords: electrochemical genosensor; human immunodeficiency virus; graphene quantum dots; gold nanoparticles; RNA detection.

Resumo

Biossensores eletroquímicos utilizando nanomateriais mostraram uma possibilidade privilegiada para o avanço dos dispositivos analíticos com potencial para diagnósticos, devido à melhora da condutividade e otimização da relação área/volume. Este trabalho apresenta a construção de um genossensor específico para o HIV através da modificação de CSPE (*carbon screen printed electrodes*) com quantum dots de grafeno decorados com l-cisteína e nanopartículas de ouro (cys-GQDs/AuNps). As Cys-GQDs foram caracterizadas pelos espectros FT-IR e UV-vis e as características eletrônicas foram avaliadas por voltametria cíclica e espectroscopia de impedância. As Cys-GQDs mostraram um efeito sinérgico com as AuNPs, melhorando a transferência eletrônica da sonda redox aniônica $[\text{Fe}(\text{CN})_6]^{3-/4-}$ na superfície do eletrodo e um aumento da área eletroativa de 37 vezes quando comparada ao eletrodo sem modificação. A detecção foi realizada de maneira *label-free* por voltametria de pulso diferencial, monitorando a resposta de corrente da sonda redox aniônica. O genossensor foi validado com amostras reais de RNA e uma curva de calibração foi construída com um limite de detecção de 1 fg/mL (1 cópia/mL). Este novo genossensor apresenta sensibilidade, especificidade, seletividade e estabilidade na detecção precoce do HIV e a plataforma baseada em cys-GQDs e AuNps melhora o desempenho geral do genossensor, que são características importantes para a aplicação comercial de biossensores.

Palavras-chave: genossensor eletroquímico; vírus da imunodeficiência humana; pontos quânticos de grafeno; nanopartículas de ouro; Detecção de RNA

1. Introduction

The first cases related to HIV infection occurred in 1981 yet, 40 years have passed and the virus responsible for causing Acquired Immunodeficiency Syndrome (AIDS) continues to be a global concern [1,2]. According to UNAIDS, since the beginning of the HIV pandemic, 84.2 million people have been infected with HIV. In 2021 is estimated that 38.4 million people live with HIV and 1.5 million were infected worldwide [3]. Even though there is no cure for AIDS, antiretroviral therapy (ART) allows the reduction of viremia, control of symptoms and increasing the life expectancy of people living with HIV [4]. Early detection and diagnosis of HIV is critical for the rapid initiation of ART [5]. The detection of HIV-related biomarkers depends on the time after the initial infection; viral RNA appears approximately 10 days after infection; afterwards, the p24 antigen may be detected, however it rapidly reduced due to the complexation of this antigen with emerging antibodies; about 4 to 6 weeks after the initial infection, antibodies begin to appear and most infected individuals are seroconverted by 3 months [6]. Serological tests detect antigens and antibodies in serum and they are mainly immunoenzymatic tests (EI), such as enzyme-linked immunosorbent assay (ELISA) testing [7]. Molecular tests (TM) are used for the detection of RNA and DNA, such as reverse transcription polymerase chain reaction (RT-PCR) [8]. ELISA-based and RT-PCR tests are considered the gold standard in HIV diagnosis, yet both have limitations. For instance the need for specialized people, the use of robust equipment, several stages in execution, large volumes of reagents and high cost rates [8,9].

Biosensors are analytical devices used to measure biological or chemical reactions by generating a signal proportional to the concentration of the analyte of interest [10]. The types of signal transducers are typically classified in optical, electrochemical and mass-based [11]. Electrochemical biosensors have had great success in biosensoriation due to sensitivity, selectivity, low cost and quick responses, especially when the detection of genetic material is required, such as the early stages of viral infection [12,13]. Biosensors that have genetic material as a biological element of recognition are known as genosensors [11]. These biological elements are usually small fragments of oligonucleotides, referred to as probes. These probes interact with DNA or RNA via pairing of nitrogen bases, known as hybridization [14]. Hybridization is highly specific and causes changes in electrical

parameters in the electrochemical platforms and generates signals that can be detected using electrochemical techniques, such as cyclic voltammetry (CV), square wave voltammetry (SWV) and differential pulse voltammetry (DPV) [15].

Electrochemical platforms stand out for their low cost, the possibility of miniaturization, selectivity and stability, especially when carbon inks are used in the construction of these platforms [16]. Carbon inks are non-metallic conductive agents that provide low detection limits. Conductive inks allow greater flexibility in electrode design and ease of application in screen printed electrodes (SPE) [17]. Therefore, all these features make carbon ink-based electrochemical biosensors market-friendly and promising in point-of-care diagnosis.

Nanotechnology is a strong ally in the development of high performance biosensors [18]. Several nanomaterials can be applied to increase the sensitivity of biosensor platforms due to unique characteristics such as size, electrical conductivity, plasmonic and quantum effects [19]. Herein, graphene quantum dots (GQDs) and gold nanoparticles (AuNps) were used to increase biosensor performance. GQDs are nanomaterials of the graphene family with zero dimension [20]. These materials are graphene sheets of less than 100 nm and have quantum effects, as well as extraordinary optical and electrical properties [21,22]. The high surface area of GQDs allows the anchoring of a large number of molecules [21]. In addition, quantum confinement and edge effects lead to rapid electron transport and excellent conductivity, making these materials ideal for signal transduction [21].

AuNps are extensively studied due to their characteristics, such as catalytic, biological, optical, electrochemical and electronic activities [23]. The interaction between analyte and AuNps may alter physicochemical properties like surface plasmonic resonance, conductivity and redox behavior; all these changes produce detectable signals [24]. Furthermore, AuNps are easily conjugated with biomolecules for the recognition of antibodies and oligonucleotides and are widely explored in strategies of immobilization of molecules in biosensors [25].

The standard methods for detecting biomarkers of HIV infection are expensive, laborious, require large equipment and large volume of reagents. Electrochemical biosensors are potential candidates to meet these criteria due to low cost, ease of mass production, sensitivity and specificity [16].

In this work, a novel nanomaterial, GQDs functionalized with L-cysteine and AuNps were produced and added onto carbon screen-printed *electrode* (CSPE), previously developed by our group [26]. The functionalization of graphite to anchor biomolecules is a challenge given the lack of reactive sites in the bidimensional structure [27].

This novel platform enabled the development of a high-performance biosensor for HIV RNA detection. The genosensor was validated with real RNA samples and showed high sensitivity, specificity and optimal shelf life.

2. Material and methods

2.1 Reagents and materials

Sulfuric acid (99%), gold (III) chloride trihydrate ($\geq 99.9\%$), graphite flakes ($> 99.9\%$) potassium ferricyanide ($\geq 99.5\%$), were purchased from Sigma-Aldrich. Disodium hydrogen phosphate ($\geq 99\%$), monosodium hydrogen phosphate ($\geq 98\%$), sodium citrate dihydrate and potassium chloride ($\geq 99\%$) were purchased from Neon. Butylglycol acetate ($> 98\%$), vinyl resin (A.P), carbon black, silver nitrate (A.P), potassium hexacyanoferrate (III) $K_3[Fe(CN)_6]$ ($>99\%$), potassium hexacyanoferrate (II) $K_4[Fe(CN)_6]$ ($> 99.5\%$), methyl isobutyl ketone (99%), L-cysteine (A.P) and ethylene glycol (A.P) were purchased from Dinâmica, Mexichem S/A, Arinos, Qhemis, Fluka Analytical, Acros Organics, Synth and Vetec respectively.

The oligonucleotides used in this work were obtained from Exxtend and prepared in a phosphate buffer (0.1 mol/L, pH 7.4). The HIV1S probe was synthesized with thiol (5'-(SH)TGGTCTTCTGGGGCTTGTTCCAT-3') and the non-complementary targets of SARS-COV-2 (5'-AATGTTTGTAACCGGCGTTT- 3') and Zika virus (5'-CTTGTGTGTTGGGCAGCGG-3') were used for the study of specificity. The selectivity and validation assays of the genosensor were conducted using gRNA from plasma samples from HIV+ and HIV- patients provided by Adolf Lutz Institute (SP). All the gRNA samples were purified using an ABBOTT m2000sp extractor and quantified by RT-PCR and by spectrophotometer (BioDrop®) prior to electrochemical detection. The use of human samples in this work is regulated by the Human Research Ethics Committee by the registration number 4348672.

2.2 Apparatus

Differential pulse voltammetry (DPV) and cyclic voltammetry (CV) were performed in CHI Instruments potentiostat (Model 760C, USA) and electrochemical impedance spectroscopy (EIS) in an Autolab potentiostat (Model PGSTAT302N, Metrohm). The CSPE was assembled according to Moço *et al.* [26] and connected to the potentiostat via a screen printed electrode adapter from DropSens. Infrared spectra of cysGQDs were performed on the Spectrum Two FT-IR Spectrometer (Perkin Elmer) and morphological analyses were performed by atomic force microscopy (AFM) on a microscope from AFM5100N Hitachi and scanning electron microscopy (SEM) on an EVO MA10 Zeiss microscope. All analyses are represented as the arithmetic mean value $\pm$ standard deviation for triplicate measurements and unpaired t-test, were carried out when applicable using graphpad. All voltammograms were subtracted from their respective baseline and plotted with Origin 7. The value for the variation of peak current oxidation (Δi_p) was measured as the peak height.

2.3 Preparation of carbon-ink based electrodes

The preparation of the inks and the construction of the electrode was carried out according to Moço *et al.* [26]. The carbon ink was printed as a working electrode and a counter electrode. Silver ink was used as a reference electrode (Ag^0). The inks were printed on PVC sheets using an adhesive mold and dried at 55 °C for 25 minutes.

2.4 AuNps and cys-GQDs synthesis

The synthesis of AuNps was performed following the Turkevich-Frens methodology [28]. This synthesis uses sodium citrate 1% (w/v) as a reducing agent of gold salts using tetrachloroauric acid 1% (w/v). The resulting nanoparticles are stabilized by citrate ions. The AuNps were stored at 4 °C until their use. The absorption spectrum in UV-Vis was analyzed in a spectrophotometer, using the dilution of 1:1 in deionized water in quartz cuvettes (1 cm optical path). This solution has an absorption peak at 520 nm and refers to AuNps from 15 to 20 nm in size.

The synthesis of GQDs functionalized with L-cysteine was performed by pyrolysis in a one-step method using ethylene glycol as a carbon source [29]. In an erlenmeyer, 0.3g of L-cysteine with 10 mL ethylene glycol were added. The solution was kept under agitation at 100°C for one hour. Initially, the solution is colorless and then develops into an orange and lastly turns into a dark yellow. The absorption spectrum in UV-Vis was analyzed in the spectrophotometer, using the dilution of 1:1 in deionized water in quartz cuvettes (1 cm optical path).

2.5 Genosensor assembly (CSPE/cys-GQDs/AuNps/HIV1S) and electrochemical detection of HIV RNA

Before use, all electrodes were subjected to CV in sulfuric acid (0.5 mol/L) solution and phosphate buffer (0.1 mol/L, pH = 7.4) between 0.00 V to +1.00 V, 20 cycles at 500 and 100 mV/s. This electropolishing procedure was made to clean the electrode surface and selects the ones with the same electroactive profile for the next experiments. The modification of the CSPE surface with cys-GQDs and AuNps was performed by drop casting. First, 3 μ L of cys-GQDs was pipetted on the electrode surface and dried at 60°C (Fig.1A). Then, 3 μ L of AuNps were also pipetted and dried at 60°C (Fig.1B). 3 μ L of thiolate probe HIV1S (10 μ g/mL in phosphate buffer 0.1 mol/L) was dripped on the electrode and kept for 1h at room temperature (Fig.1C). After this period, the electrodes were washed in a phosphate buffer for 6 seconds.

The gRNA samples from the patients were enriched with healthy plasma (1:1, v/v), prior to electro detection, and then 3 μ L (10 μ g/mL) of gRNA-enriched plasma was pipetted on the electrode and left in a humid chamber at 55 °C (Fig.1D). After 20 minutes, the electrodes were washed in phosphate buffer for 6 seconds and proceeded to detection by DPV. DPV was conducted (Fig. 1E) before and after the hybridization (modulation amplitude: 25 mV, pulse interval: 0.2 s, 50 mV/s) using the anionic redox probe $[\text{Fe}(\text{CN})_6]^{3-/4-}$ (5 mmol/L), in KCl solution (0.1 mol/L) between -0.40 and +0.80 V.

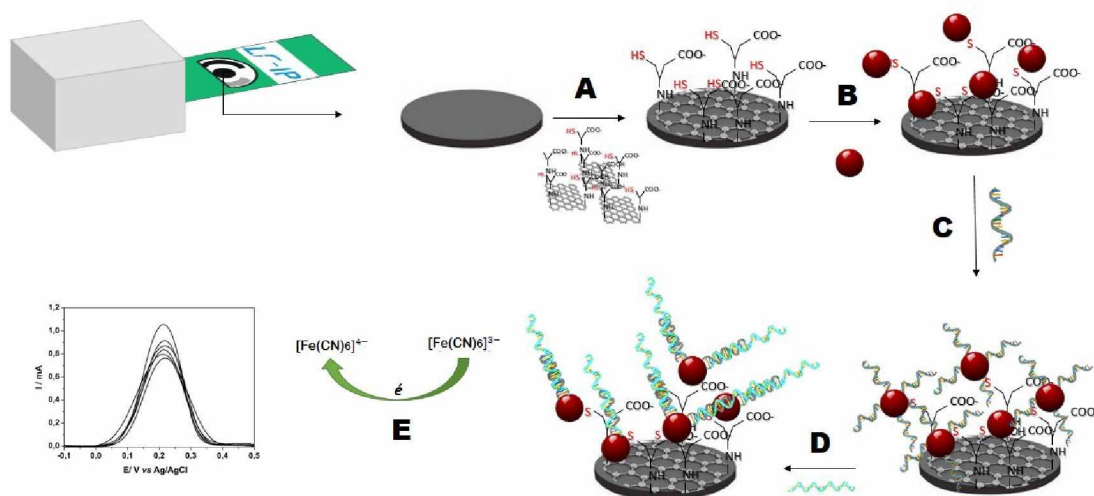


Fig. 1. Surface modification with cys-GQDs **(A)** followed by AuNps **(B)**. Immobilization step with thiolated DNA probe **(C)**. Plasma-enriched HIV RNA **(D)**. Monitoring of hybridization through the current change of the anionic redox probe $[\text{Fe}(\text{CN})_6]^{3-/4-}$ by DPV **(E)**.

2.6 Performance of the genosensor

The genosensor calibration curve was constructed by varying the concentration of gRNA samples at 10 $\mu\text{g/mL}$, 1 $\mu\text{g/mL}$, 10 ng/mL , 1 pg/mL and 1 fg/mL . These samples correspond to 1000000, 10000, 1000, 100 and 1 copies/mL, respectively. The genosensor was validated using 4 HIV+ and 2 HIV- patient samples, previously detected and quantified by RT-PCR. The stability of the genosensor was evaluated after storage at room temperature for 60 days, using probe-modified electrodes (CSPE/cys-GQDs/AuNps/HIV1S).

3. Results and discussion

3.1 Characterization of cys-GQDs by FT-IR and UV-vis spectra

The FT-IR was used to characterize the functional groups of cys-GQDs. In Fig. 2A is possible to observe the bands at 3321, 2961, 1636, 1038, 621 and 576 cm^{-1} . These bands can be attributed, respectively, to: stretch vibration of the

hydroxyl group (O-H), alkane (C-H), alkene (C=C), amine (C-N) and sulfide (C-S) [29-32].

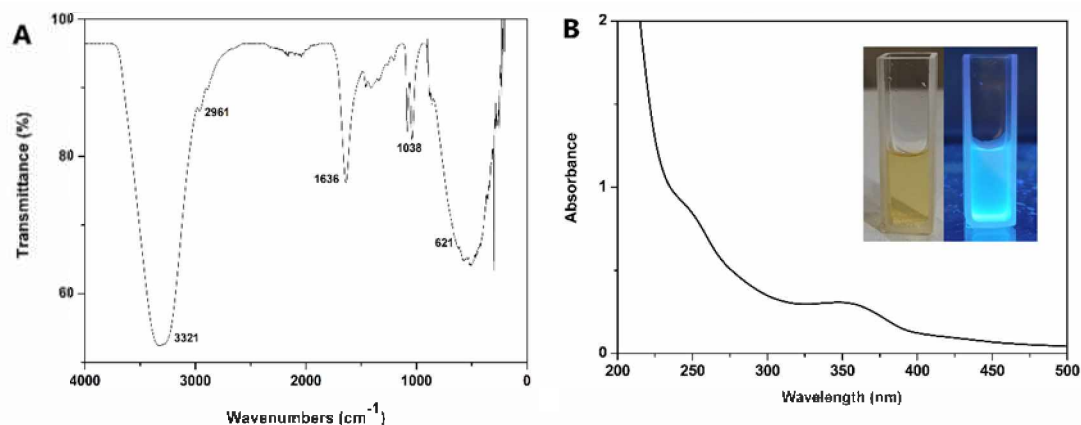


Fig. 2. FT-IR **(A)** and UV-Vis **(B)** spectra of cys-GQDs. Inset: cys-GQDs photographs under visible light and UV irradiation conditions ($\lambda_{\text{ex}}=365\text{nm}$).

These results show that cys-GQDs have many functional groups that contribute to the hydrophilic and stability in aqueous solution, besides providing the binding sites for gold nanoparticles. UV-vis spectroscopy analysis (Fig. 2B) shows a peak at 350 nm and a shoulder at 225 nm. The shoulder can be attributed to the π to π transition of C=C bonds in graphene sheets and the peak to the n to π transition of C=O bonds in cys-GQDs [29,33,34]. Photographs of the aqueous solutions of cys-GQDs are presented in the inset of Fig. 2B and show the yellow coloration and strong blue fluorescence under UV light, which is a fundamental characteristic of QDs.

3.2 Characterization of the CSPE/cys-GQDs/AuNps platform

The analysis of the electronic transfer in the electrode-solution interface was performed with redox pair $[\text{Fe}(\text{CN})_6]^{3-/4-}$. Fig. 3A. shows a progressive increase in CSPE/cys-GQDs and CSPE/cys-GQDs anodic and cathodic peak currents when compared to CSPE, meaning that electronic transfer of the redox pair occurs more easily. The electric charge (Fig.3A – Inset) increased by 2x with the modification of

cys-GQDs and 2.7x with cys-GQDs/AuNps when compared to the electrode without modification. Thus, cys-GQDs showed a synergistic effect with AuNps, improving the electronic transfer of the redox pair on the electrode surface. Metallic nanoparticles are highly conductive and the arrangement of the carbon atoms in graphene contributes to greater electron movement [20,23]. GQDs are composed of graphene fragments that have a hexagonal arrangement of carbons which provides delocalized electrons that move freely and virtually without resistance, causing a high conductivity [35].

CV profiles (Fig. 3B) in phosphate buffer of the electrode show an increase of 26x, when compared to CSPE/cys-GQDs, and 37x higher in CSPE/cys-GQDs/AuNps. The facilitation of electric transport is also observed by EIS (Fig. 3C): the resistance to charge transfer value (R_{CT}) decrease 5.5x and 29x when the bare CSPE ($1.77 \pm 0.06 \text{ k}\Omega$) is compared with the CSPE/cys-GQDs ($0.32 \pm 0.02 \text{ k}\Omega$) and the SPCE/ CSPE/cys-GQDs /AuNps ($0.06 \pm 0.004 \text{ k}\Omega$). The increase in the capacitance of the electrode is possibly due to the accumulation of charge at the electrode surface, which is accompanied by a decrease in resistance to load transfer, allowing the electronic exchange to occur more easily. These data show the efficiency of cys-GQDs and AuNps as electron transporters which causes a greater interaction between the electrode and analyte and increases the electrochemically active surface area [21].

Scanning electron microscopy (SEM) images show an increase in the homogeneity of globular structures in each modification step (Fig.3D). In the electrode only with carbon ink (Fig.3D-I), there are more cracks and globular structures, while the addition of cys-GQDs (Fig.3D- II) and AuNps (Fig.3D-III) promotes a smoothing of the surface. Thus, the modification is clear at each step and there is no leaching of the material deposited on the surface of the electrode.

In the development of high performance biosensors it is crucial to explore materials that amplify the signal, allowing the tracking of low amounts of analyte. The interaction between graphene and gold has great signal amplification capacity and can be described as nanozymes, a recent term that refers to nanomaterials that mimic the properties of enzymes like the catalytic power [36]. The interaction between carbon ink and cys-GQDs can occur via π - π stacking and the interaction

between cys-GQDs and AuNps probably occurs mainly via covalent bond between gold and sulfur. However, since AuNps are stabilized by citrate, negative charges may interact electrostatically with positive nitrogen charges [37].

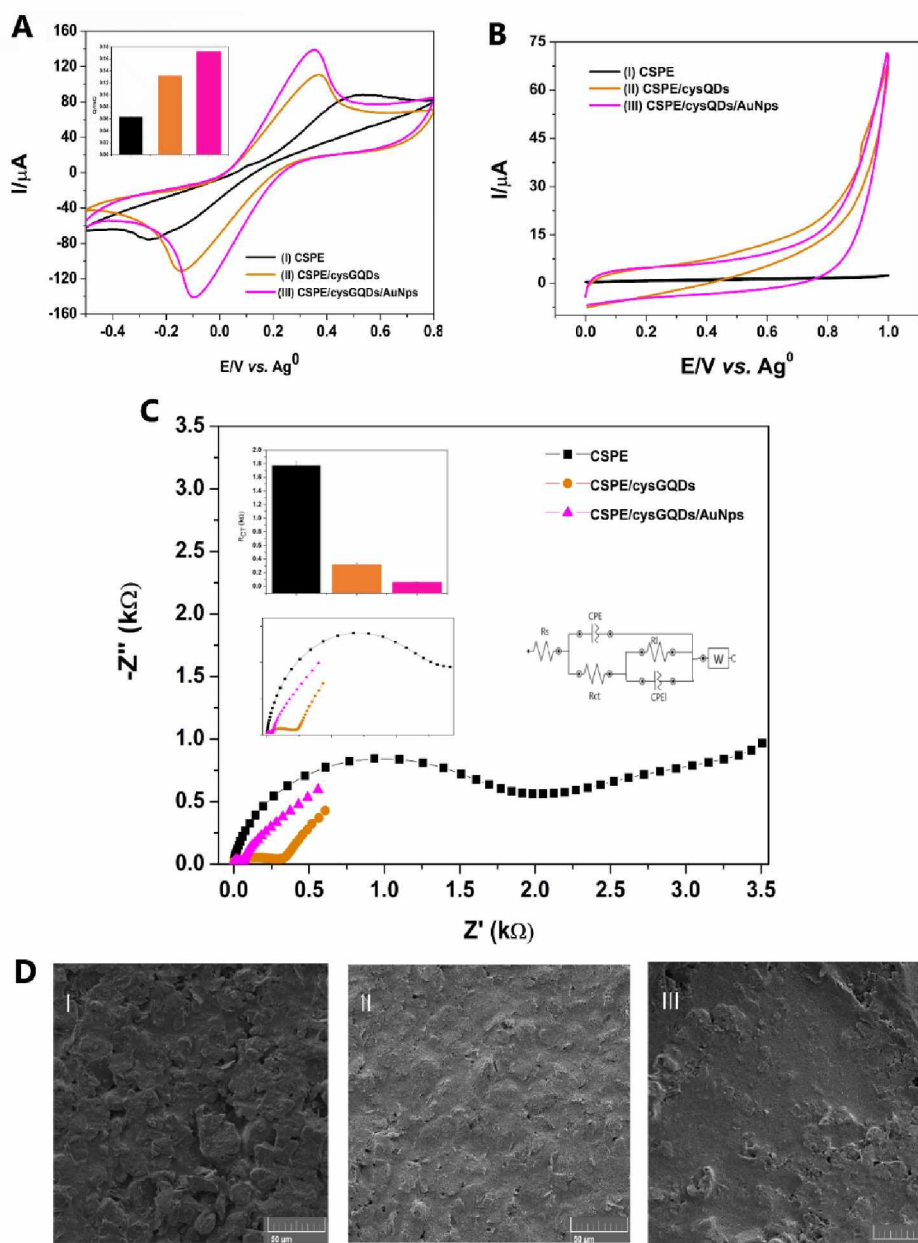
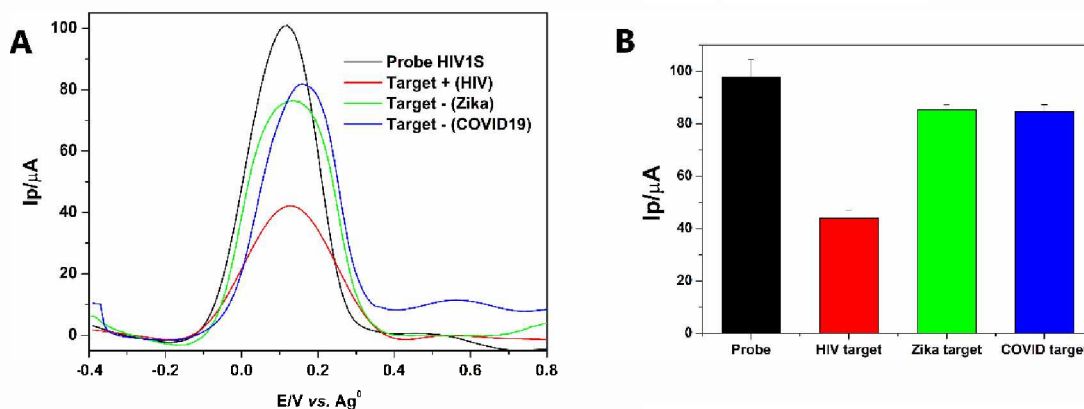


Fig. 2. (A) Cyclic voltammograms with anionic redox probe: bare CSPE (I), CSPE/cys-GQDs (II) electrode, and CSPE/cys-GQDs/AuNps electrode (III). Inset: histograms of the electrical charge involved in the oxidative and reductive processes. Electrolyte: $[\text{Fe}(\text{CN})_6]^{3-/4-}$ (5.0 mmol/L) solution containing KCl (0.10 mol/L), $\nu = 50 \text{ mV/s}$. (B) Cyclic voltammograms with phosphate buffer: bare CSPE

(I), CSPE/cys-GQDs (II) electrode, and CSPE/cys-GQDs/AuNps electrode (III). Electrolyte: phosphate buffer (0.10 mol/L), pH 7.4, $\nu = 30$ mV/s. **(C)** Nyquist plots from electrochemical impedance spectroscopy in KCl solution (0.1 mol/L) containing $[\text{Fe}(\text{CN})_6]^{3-/4-}$ (5 mmol/L) for bare CSPE (■), CSPE/cys-GQDs (●) and CSPE/cys-GQDs/AuNps electrode (▲). EIS conducted in open circuit, frequency ranging from 10 kHz to 0.1 Hz and amplitude of 10 mV. Full lines represent fitting from equivalent circuit simulation. Inset: bar chart of R_{CT} from EIS of bare CSPE, CSPE/cys-GQDs and CSPE/cys-GQDs/AuNps and amplification of the Nyquist plots. **(D)** SEM images of bare CSPE (I), CSPE/cys-GQDs (II) and CSPE/cys-GQDs/AuNps (III).

3.3 Functionalization and Detection of the HIV RNA

The AuNps, in addition to increasing the surface area of the modified electrode (CSPE/cys-GQDs), allows anchoring of the probe biomolecule HIV1S probe via Au-S bond. This covalent bond is formed after dissociation of S-H followed by formation of the Au-S bond [38]. The nitrogen bases of the probe may also interact with gold, but the Au-S bond occurs preferably, which ensures the orientation of DNA in the AuNps through the 5'-SH end of the probe [39].



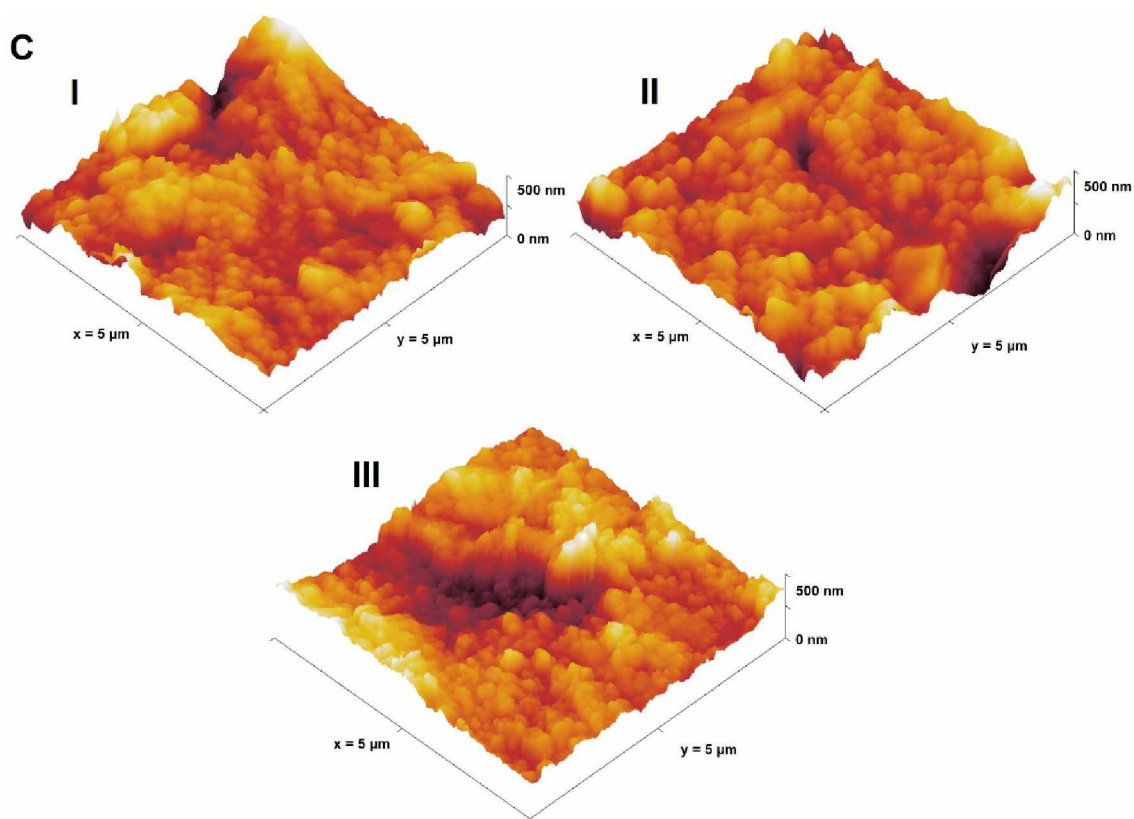


Fig. 3. (A) DPV of the anionic redox probe $[\text{Fe}(\text{CN})_6]^{3-/4-}$ in the presence of HIV probe, complementary target (HIV) and non-complementary (Zika and SARS-COV-2). **(B)** Bar chart of the values of the current peaks with probe, positive target (HIV) and negative targets (Zika and SARS-COV-2). Electrolyte: $[\text{Fe}(\text{CN})_6]^{3-/4-}$ (5.0 mmol/L) solution containing KCl (0.10 mol/L), modulation amplitude: 25 mV, pulse interval: 0.2 s, $\nu = 50$ mV/s. **(C)** AFM images of the modified electrode CSPE/cys-GQDs/AuNPs with probe (I), HIV positive target (II) and SARS-COV-2 negative target (III).

The DPV profile of the anionic redox probe in the presence and absence of the complementary target are shown in Fig. 4A. When the complementary target (HIV) is added, there is a reduction of the oxidation peak current (I_p) of 2.2x (from $97.8 \pm 6.8 \mu\text{A}$ to $43.9 \pm 2.9 \mu\text{A}$, +0.13V, Fig 4B) in relation to the HIV probe. The current values of the negative targets (SARS-COV-2 and Zika virus) are respectively $84.62 \pm 2.6 \mu\text{A}$ and $85.12 \pm 2.1 \mu\text{A}$, similar current values to the probe, showing the specificity of the genosensor. Some studies have reported the mechanism of the anionic redox probe in genosensors [40-42]. The transfer of electrons between the

anionic redox probe and the electrode surface is hindered when the biorecognition occurs between the DNA probe and the positive target, as hybridization leads to the formation of double-strand DNA, which is less flexible than the single-strand DNA, causing a decrease in electron transport and consequent reduction of the peak current of the anionic redox probe.

Morphological changes onto the surface of the electrodes with the addition of different targets can be observed by AFM (Fig. 4C) and changes in the root mean square roughness coefficient (Rq). The electrodes with DNA probe (Fig.4C-I), HIV positive target (Fig.4C-II) and SARS-COV-2 negative target (Fig.4C-III) presented Rq of 135.6 ± 18.3 , 158.7 ± 13.6 and 129.6 ± 15.6 nm, respectively. After hybridization with the probe, roughness increases because of the specific interaction between the probe and HIV RNA and globular structures are formed, as double strand nucleic acids are more stiff and less flexible than single-strand DNA [40]. On the other hand, the roughness of the negative target surface was similar of the probe. Thus, the AFM analysis corroborates the electrochemical studies.

3.4 Calibration curve, storage stability and genosensor validation

The current response profile of the redox probe $[\text{Fe}(\text{CN})_6]^{3-/4-}$ with different concentrations of gRNA is shown in Fig. 5B. The lower the concentration of the complementary target, the higher the response, since fewer hybridization events occur and allows the redox probe to reach the electrode more easily. Thus, there was a logarithmic relationship ($I_p (\mu\text{A}) = 29.8 (\mu\text{A/g/mL}) \cdot \log[\text{gRNA}] (\text{g/mL}) + 2.80 (\mu\text{A})$, $r = 99$) between the concentration of gRNA and the current peak (Fig. 5A) ranging from 10 $\mu\text{g/mL}$ to 1 fg/mL (1000000 to 1 copies/mL). The obtained P value (< 0.0001) was extremely statistically significant.

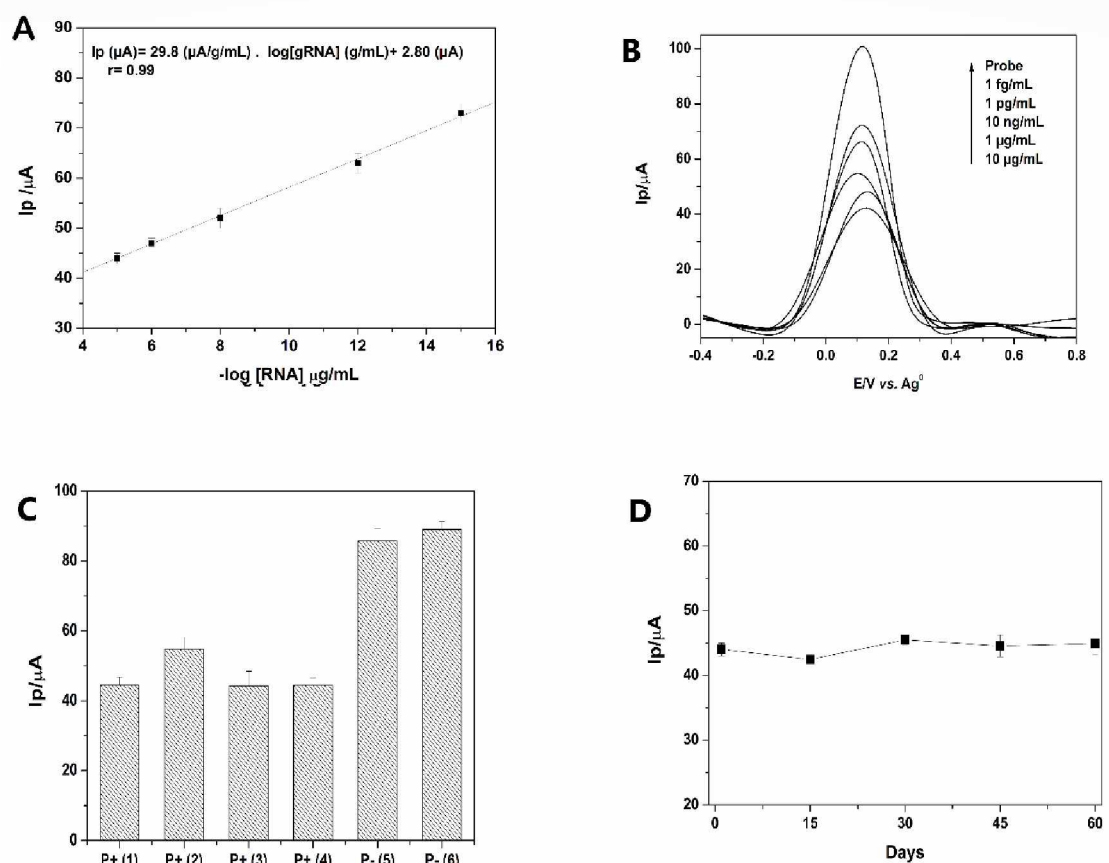


Fig. 4. (A) Calibration curve of the RNA HIV genosensor. **(B)** Differential pulse voltammograms of the redox probe with different target concentrations. **(C)** Bar chart of peak current (I_p) for RNA samples from people living with HIV (1-4) and sample of people who tested negative for HIV (5,6) **(D)** Storage stability at room temperature. Electrolyte: $[Fe(CN)_6]^{3-/4-}$ (5.0 mmol/L) solution containing KCl (0.10 mol/L), modulation amplitude: 25 mV, pulse interval: 0.2 s, $v = 50$ mV/s. For the calibration curve P value < 0.0001.

Genosensor validation was carried out with 4 RNA samples from people living with HIV (HIV +) and 2 negative samples (HIV -). All samples were enriched with healthy plasma (1:1). As can be seen in Fig. 5C, all positive samples differed from the negative and were quantified previously by RT-PCR.

The stability of the response of biosensors to time is a very important parameter for future commercial use, since it refers to shelf life. The present genosensor maintained 100% of the response for 60 days (Fig.5D), stored at room temperature and in closed containers. Most forms of storage involve the use of

cooling (4°C) [43-48], consequently adding a step that make the maintenance of biosensors more costly. Table 1 lists other genosensors that also utilized GQD in the assemble, along their analytical performance and stability. Some studies do not report stability tests, and some cases up to a couple weeks or days.

To date, there are no genosensors for detecting HIV RNA based on GQDs as an electrochemical platform in the literature. Gogola *et al.*, [47] recently developed an aptasensor for HIV p24 protein detection with a detection limit of 51.7 pg/mL. They used GQDs synthesized by citric acid pyrolysis as platform signal amplifiers. Kaimal *et al.*, [43] developed a genosensor using the GQD interface to detects the guanine and adenine nitrogen bases with detection limits of 1.71 and 1.84 μM , respectively. Most recent biosensors that use GQDs on electrochemical platforms using DNA as a biorecognition mode are aptasensors and it mainly detect proteins and chemical compounds (Table 1). The HIV RNA is an important biomarker for infection diagnosis and monitoring, and the development of biosensor platforms that are selective, specific and stable it is crucial for the point-of-care use.

Table 1. Recent examples of GQDs-based electrochemical genosensors and aptasensors.

Technique	DLR	DL	Sample volume	Target	Stability	Ref
DPV	4 to 4 × 10 ⁵ fM	1.2 fM	5 μL	Peptide	4 weeks	[45]
DPV	0.35 to 100 μM	0.1 μM	10 mL	Chemotherapy compound	Not reported	[49]
CV/DPV	2.3 to 6.1 nM	0.091 pM	200 μL	miRNA	Not reported	[50]
DPV	10 ⁻¹⁶ – 10 ⁻¹¹ M	3.8×10 ⁻¹¹ M	20 μL	Organophosphate pesticide	3 weeks	[46]
CV/DPV	0.5 to 20 mM / 0.1 to 20 mM	1.71 μM 1.84 μM	10 μL	DNA	5 weeks	[43]

DPV	10^{-15} M to 10^{-10} M	10^{-15} M	10 μ L	Organophosphate pesticide	Not reported	[51]
EIS	1 to 10 ng/mL	6.0 pg/ mL	10 μ L	Protein	96h	[44]
CV	0.93 to 93 mg/ml	51.7 pg mL ⁻¹	Not reported	Protein	4 weeks	[47]
DPV	1.0 to 1×10^5 fM	0.37 fM	5 μ L	Organic compound	Not reported	[48]
EIS	1 to 10 ng/mL	0.00141 ng/mL.	10 μ L	Protein	4 days	[52]
DPV	10 μ g/mL – 1 fg/mL	1 fg/mL	3 μ L	RNA	60 days	This work

LD: limit of detection; DLR: detection linear range.

4. Conclusions

Introduction of novel nanomaterials signify a foremost opportunity for the development biosensor platforms. This study showed the application of GQDs functionalized with L-cysteine and AuNPs in carbon ink electrodes and the remarkable synergistic effect on conductivity, where synthesis of GQDs was performed in a simple way in a one-step method. Electrode detection was performed by DPV in a label-free manner, monitoring the peak oxidation current of anionic redox probe $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$. The genosensor was specific, highly sensitive (1 copy/mL - 1 fg/mL), selective and maintained its response for 60 days at room temperature.

The genosensor was validated with real RNA samples in plasma and proved to be very efficient. This work showed a new genosensor for HIV RNA detection using GQDs as electrochemical signal amplifiers with market potential, using low-cost reagents, ready-to-use electrodes and in an easy way.

CRedit author statement

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Conflict of interest statement

The authors declare no conflict of interest.

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ANEXOS



Carbon ink-based electrodes modified with nanocomposite as a platform for electrochemical detection of HIV RNA

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ABSTRACT

This work reports a new and easy-to-use methodology for construction of screen-printed electrodes (SPE) based on carbon ink and its modification with 4-aminothiophenol (4-ATP) and gold nanoparticles for detection of HIV RNA. Morphological analysis for SPE/4-ATP/AuNPs-DNA probe showed increased roughness indicating that 4-ATP monolayer works as a network for connection of functionalized gold nanoparticles with specific oligonucleotide probe to the RNA target. The biosensor response was monitored by indirect electrodedetection using ethidium bromide as indicator of duplex formation. The peak current response of ethidium bromide was linear related to the logarithm of concentration of HIV RNA, detecting up to 5 copies/mL and linear range of 6 to 11,000 copies/mL of RNA, covering the clinically relevant concentration range (50–200 copies/mL). The biosensor discriminates negative and positive samples, making it possible to quantify the viral load, a crucial factor for the development of biosensors for the early detection of HIV.

1. Introduction

Human Immunodeficiency Virus (HIV) was initially reported in the early '80s, since then, it affects generations despite enormous advances in testing and treatment. According to the Joint United Nations Program on HIV/AIDS (UNAIDS), in 2019, 38 million people were affected by HIV [1]. HIV is a lentivirus of the *Retroviridae* family, having in common the genome composed of RNA that replicates through reverse transcriptase (RT). Continuous replication leads to immunological impairment through the destruction of CD4 lymphocytes. The destruction of immune system cells causes the appearance of opportunistic infections, such as pneumonia and tuberculosis, defining the Acquired Immunodeficiency Syndrome (AIDS) [2]. The success of antiretroviral treatment is dependent on early detection and continuous monitoring of viral load in affected individuals [3]. HIV detection at the beginning of infection leads to early initiation of antiretroviral treatment, which improves life's quality of individuals living with HIV, since they help reduce viral load and consequently strengthen the immune system, a recurrent target of the disease [4].

The diagnosis of HIV is performed by serological and molecular methods; the first one aims to detect antibodies against HIV-1–2 and p24 antigens, namely Enzyme-Linked Immunosorbent Assay (ELISA) and Western Blot; the second methodology detects nucleic acids mainly by amplification technology, such as Reverse Transcription Polymerase Chain Reaction (RT-PCR) [5]. Until 10 days from the onset of infection, called the “eclipse period”, there are still no commercially available serological and molecular tests. Nucleic acid-based methods can detect DNA or RNA at the end of the eclipse period and serological methods detect p24 protein after 14 days [6]. Twenty-one days after the onset of infection it is possible to use serological methods for detection of antibodies and this period, between the first day until 21 days, is called “acute infection” and the virus detection is only possible by nucleic acids and protein p24 detection [5]. However, molecular diagnostic methods present several disadvantages, such as the high cost of commercial kits, which is particularly harmful in countries with limited public health resources [7]. In addition, the high level of automation required, as well as the need for specialized people, constitute a barrier in the early, rapid and inexpensive HIV detection, interfering in the rapid start of

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antiretroviral therapy [8].

The term “ASSURED tests” was coined to represent an ideal diagnostic test for areas with limited resources for diagnosis of major epidemics, namely accessibility, sensitivity (low rate of false negatives), specificity (low rate of false positives), simple performance which requires little training, speed, robustness, free of equipment and able to reach those in need [9]. In this context, biosensors, along with microfluidics and nanotechnology, can meet these criteria, since they require small volume of samples, easy data interpretation and a rapid and early diagnosis to the ones in need [10].

Electrochemical biosensors stand out due to the possibility to develop low-cost microelectronic circuits and miniaturization [11]. The efficient joining of signal transduction and biorecognition components enables the development of several platform configurations for sensitive and selective detection of the target analyte, starting from the creation of new materials for biomolecules immobilization to new forms of electrochemical detection [12]. There are numerous components of HIV that can be detected through redox changes on the surface of an electrode. The components work as biomarkers in the detection of the disease, such as those directly linked to HIV, namely viral protein p24 [13] and viral RNA [14], as well as components of the immune system, antibodies and TCD4 lymphocytes [15], for example.

Nucleic acids are biomolecules with desirable intrinsic characteristics to biorecognition processes in biosensors, since they present a sequence-specific hybridization behaviour with their complementary target [16]. The electrical properties of both single-strand DNA (ssDNA or RNA) and double-strand DNA (dsDNA) change before and after the hybridization process [17]. This change is detectable in terms of electron transfer by electrochemical techniques, such as cyclic voltammetry (CV), square wave voltammetry (SWV) and differential pulse voltammetry (DPV), by monitoring the oxidation and/or reduction of nucleic acids bases (label-free detection) or using labels to monitor the hybridization process [11].

The application of screen-printed carbon electrodes (SPCE) for biosensing is widely studied [18,19]. They consist of high portability and market-friendly devices, being applied to a low cost and facile production on a wide scale, for example, the commercially well-established glucose biosensor [20]. Analytical parameters such as sensitivity and selectivity of SPCE-based platforms can be increased with the use of materials like conductive polymers and nanomaterials [18]. In addition, important factors to overcome barriers between the applicability of scientific development and commercial use, namely stability and reproducibility can be achieved through the association between electrochemical and nanomaterial biosensors [21,22].

The 4-aminothiophenol (4-ATP) is an organic molecule with electrochemical and optical applications due to its characteristics such as the ability to form self-assembled layers (SAMs), forming nanomaterial networks and it works as a bifunctional agent [23]. The thiol group of its structure makes it a versatile material, mainly in methodologies involving gold electrodes and gold nanoparticles (AuNps) [24]. AuNps have a high surface-to-volume ratio and high surface energy to provide a stable immobilization of a large amount of biomolecules and retention of their bioactivity, also reflecting on better results in stability, reproducibility and sensitivity [22].

Herein, due to the lack of accessible ASSURED HIV diagnosis, this work reports a facile methodology to construct screen-printed carbon electrode (SPCE) in a manufactured way and its application in a novel platform using SPCE modified with 4-ATP and AuNps conjugated with a specific DNA capture probe to offer a low cost, selective and sensitive alternative for electrochemical biosensing to detect the genomic RNA of HIV in human plasma.

2. Material and methods

2.1. Reagents and materials

Sulfuric acid (99%), gold (III) chloride trihydrate ($\geq 99.9\%$), graphite flakes ($> 99.9\%$) potassium ferricyanide ($\geq 99.5\%$), hexammineruthenium (II) chloride $\text{Ru}(\text{NH}_3)_6\text{Cl}_2$ (98%), 4-aminothiophenol (97%), ethidium bromide ($\geq 99\%$) were purchased from Sigma-Aldrich. Disodium hydrogen phosphate ($\geq 99\%$), monosodium hydrogen phosphate ($\geq 98\%$), sodium citrate dihydrate and potassium chloride ($\geq 99\%$) were purchased from Neon. Butylglycol acetate ($> 98\%$), vinyl resin (A.P), carbon black, silver nitrate (A.P), potassium hexacyanoferrate (III) $\text{K}_3[\text{Fe}(\text{CN})_6]$ ($> 99\%$), potassium hexacyanoferrate (II) $\text{K}_4[\text{Fe}(\text{CN})_6]$ ($> 99.5\%$), methyl isobutyl ketone (99%) were purchased from Dinâmica, Mexichem S/A, Arinos, Qhemis, Fluka Analytical and Acros Organics, respectively.

The design for the printed electrode was created in Corel Draw 2018® and the molds were printed with a Roland® 45° needle on the vinyl adhesive with dimensions of 210 mm by 297 mm (dimensions of an A4 sheet). These dimensions cover the space needed to print 63 templates electrodes. The printed substrate for the manufactured SPCE was polyvinyl chloride (PVC). Two vinyl adhesive templates were used in the construction of the SPCE. The first one contains the template design of the working, counter, reference electrodes and the wires/tracks that connect them to the electrochemical device. The second adhesive is placed at the end of the print on top of the electrode and demarcates the electroanalytical area.

2.2. Apparatus

The SPCE has been connected to the electrochemical workstation via DropSens screen printed electrode adapter. All electrochemical techniques of this work were performed in Autolab potentiostat (Model PGSTAT302N, Metrohm) with NOVA software. The surface morphology of the electrodes were assessed by scanning probe microscopy (Model S100 N, Hitachi), performed in the dynamic force microscope (DFM) mode, and by scanning electron microscopy (SEM) on an EVO MA10 Zeiss microscope.

2.3. Preparation of carbon and silver inks

Carbon conductive ink was prepared to be printed as a working electrode and counter electrode (Fig. 1a). The silver ink was printed as a reference electrode (Ag^+). Silver ink was prepared using two solutions, one with 10 g of silver flake, produced according to Paula *et al.*, [25], in 1.9 mL of butylglycol acetate and another composed of 10 mL of vinyl resin 20% in methyl isobutyl ketone. The second solution was heated to 50 °C under agitation until its complete solubilization. Once solubilized, 1.022 g of this solution was added to the first prepared solution containing silver flakes. The mixture was kept in constant agitation and heating until its homogenization.

For the carbon ink, first, 1.6 g of vinyl were solubilized in 11 mL of methyl isobutyl ketone with heating at 50 °C under constant agitation. After complete solubilization, 7.3 g of graphite flakes (325 mesh), 1.6 g of carbon black, and 9.0 mL of butylglycol acetate were added. Then, agitation was maintained at 50 °C until the ink was completely homogenized. Both silver and carbon ink were stored at 8 °C.

2.4. SPCE/ Ag^+ construction

The first step for SPCE/ Ag^+ construction is glueing the first vinyl adhesive on PVC of 5.0 mm thickness. This vinyl adhesive contains the template of the electrode track and where the paints will be printed (Fig. 1a). Thus, the adhesive is removed and exposes the path of each electrode. Both inks are printed with a rectilinear spatula and kept at 55 °C for 25 min for complete drying (Fig. 1b). Finally, the first adhesive

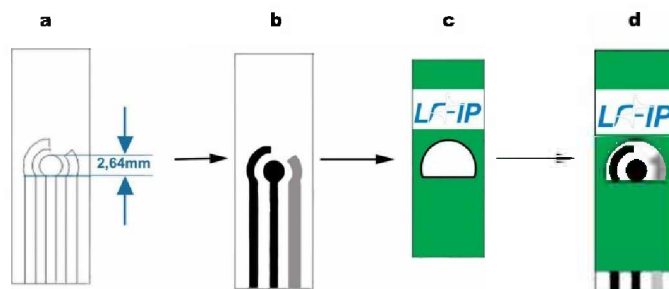


Fig. 1. SPCE/Ag⁺ construction. (a) First template vinyl adhesive with working, counter and reference electrodes tracks. (b) After removing the adhesive tracks, carbon and silver inks are printed with a spatula. (c) Second template adhesive is used to isolate and delimit the electroanalytical area. (d) Final SPCE/Ag⁺ construction.

template is entirely removed and another one is placed on top of the electrode (Fig. 1c), then, only the working, reference and counter electrodes are exposed for future experiments (Fig. 1d).

2.5. Gold nanoparticle synthesis

The AuNps synthesis method was based on the Turkevich-Frens methodology [26,27]. Basically, in this method, sodium citrate is used to reduce gold salts, which leads to the nucleation and growth of gold nanoparticles with a size of 15 to 20 nm, stabilized by citrate ions. Subsequent to the synthesis, the AuNps were stored at 4 °C until their use. The absorption spectrum was analyzed in a UV-VIS spectrophotometer in quartz cuvettes (1 cm optical path). A 1:1 dilution of AuNps in deionized water was prepared and the spectrum was analyzed at 300 to 700 nm to observe the peak absorption at 520 nm, wavelength that refers to gold nanoparticles with sizes between 15 and 20 nm.

2.6. SPCE/4-ATP/AuNps-HIV1S platform

The electrodes produced (Fig. 2a) undergo electrochemical polishing through the CV technique and the electrodes with reproducible electrochemical profiles are selected for the later stages. Electropolishing is performed in 0.5 M sulfuric acid solution and phosphate buffer (0.1 mol/L, pH 7.4) between 0.00 V to + 1.00 V, 20 cycles at 500 and 100 mV/s.

The thiolate oligonucleotide sequence HIV1S (5'-SHAATTGGAGAGCAATGGCTAGTGA-3') used in this work [28] as the probe was purchased from Invitrogen and prepared in a phosphate buffer (0.1 mol/L, pH 7.4). The evaluation of the platform's selectivity was carried out using Zika virus genomic RNA (gRNA) and *Staphylococcus aureus* bacteria genomic DNA (gDNA) as negative control under the same conditions as the positive HIV gRNA (positive target). All the gRNA samples were purified from plasma samples using an ABBOTT m2000sp extractor and quantified by RT-PCR at the Adolf Lutz Institute (SP) and by spectrophotometer (BioDrop®) prior to electrochemical detection. The use of human samples in this work is regulated by the Human Research Ethics Committee by the registration number 4348672.

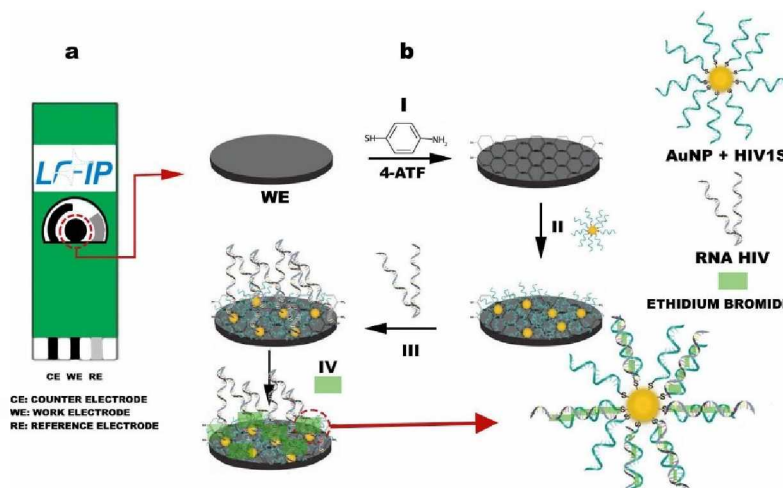


Fig. 2. Schematic illustration of SPCE/4-ATP/AuNps-HIV1S platform construction. (a) SPCE/Ag⁺. (b) Sequence of the modifications on the working electrode surface. I: monomeric modification with 4-aminothiophenol. II: addition of the previously functionalized AuNps with the thiolate capture probe HIV1S. III: Plasma-enriched HIV viral RNA. IV: DNA intercalant used as an indicator of the hybridization reaction.

The HIV-specific probe (HIV1S) contains a thiol group at the 5' end of the oligonucleotide sequence for interaction between this group and AuNps. Thus, 2 μ L of HIV1S (10 μ g/mL, phosphate buffer 0.1 mol/L, pH 7.4) in 200 μ L of AuNps were added to occur the functionalization of AuNps with the probe. This interaction occurs at room temperature for 1 h.

For surface modification with the 4-ATP monomer, 5 μ L of 5 mM in sulfuric acid 0.5 M was dripped on the electrode (Fig. 2b – I) and left for 1 h at room temperature. Subsequently, the electrode was washed in deionized water for 6 s. Then, the DPV was performed in phosphate buffer with modulation 0.07 V, pulse interval 0.20 s and velocity of 0.03 V/s between + 0.20 and + 0.90 V. This potential window covers the oxidation of EB [(3,8-diamino-5-ethyl-6-phenyl phenatridinium bromide)] that was used as an indicator of hybridization reaction between the probe (HIV1S) and its target, the complement in the RNA molecule of the HIV virus (Fig. 2b – III and IV). Thus, the baseline resulting from this step is extracted from the final result after interaction of the EB with the target probe.

After surface modification with 4-ATP monomer, 5 μ L of the AuNps-HIV1S probe (Fig. 2b – II) were added in the electrode and left at room temperature for 30 min. After this period, the electrode was washed with phosphate buffer for 6 s.

In parallel, CV in the presence of anionic redox probe $\{K_3[Fe(CN)_6]/K_4[Fe(CN)_6]\}$ and cationic probe $\{[Ru(NH_3)_6]Cl_2\}$ (5.0 mmol/L) containing KCl (0.10 mol/L), $v = 50$ mV/s was performed to analyze the properties of electronic transfer in the electrode-solution interface comparing bare SPCE, SPCE with the 4-ATP monomer and SPCE with 4-ATP and AuNps.

Peak data were corrected by subtracting a straight line tangent to the base, adapting vertically and their characteristics (area and height) were calculated by the peak analyzer tool, included in the Origin 8 software. The peak current (I_p) was obtained by the necessary height in the graphs of current per electric potential and the electrochemical charge (Q) was obtained from the area under the curve integrating the area under the curve of the voltammograms, based on the graphs of current per time.

2.7. Electrochemical detection of HIV RNA

After the bioelectrode construction (SPCE/4-ATP/AuNps-HIV1S), gRNA of the HIV virus was detected. All viral RNA samples from infected patients were enriched with diluted plasma from healthy patients (1:10, V/V) in phosphate buffer (0.1 mol/L, pH 7.4) in order to mimetize the natural environment in which viral RNA is found in the body. 5 μ L of enriched plasma were dripped on the electrode and left at 55 °C for 20 min. After, the electrode is washed in phosphate buffer for 6 s to remove biomolecules that have not interacted with the probe. Ethidium bromide (EB) at 0.1 mg/mL, 5 μ L were added in the electrode for 10 min at room temperature and then washed in deionized water for 6 s. DPV was performed in phosphate buffer with the same parameters described in item 2.6.

2.8. Figures of merits of biosensor

Performance of the genosensor was evaluated by variation of the concentrations of HIV gRNA plasma-enriched (1:10, V/V). Biosensor calibration curve was constructed with gRNA concentrations of 6, 56, 560, 1100 and 11,000 copies/mL (Cp/mL), diluted 1:10000, 1:1000, 1:100, 1:50 and 1:5 in plasma, respectively.

Biosensor stability was evaluated after storage at 8 °C for 50 days using bioelectrodes (SPCE/4-ATP/AuNps-HIV1S) stored hermetically sealed with nitrogen gas (N_2) with (wet-chamber) and without (dry-chamber) humidity in the environment.

3. Results and discussion

3.1. Characterization of the SPCE platform

In the presence of the anionic redox pair (Fig. 3a) there was an increase in the anodic and cathodic peak currents (I_{pa} and I_{pc} , respectively) in SPCE, SPCE/4-ATP, SPCE/4-ATP/AuNps, respectively. Compared to bare SPCE, I_{pc} and I_{pa} increase 2.8x and 3.5x in SPCE/4-ATP, SPCE/4-ATP/AuNps, respectively. When analyzing the electrical charge (Fig. 3a inset), there is an increase of 2.14x and 2.76x in SPCE/4-ATP and SPCE/4-ATP/AuNps, compared to bare electrode. Therefore, it is observed that the progressive change in surface modifications occur in order to facilitate the electronic transfer of the redox pair, suggesting that the 4-ATP film have a positive net charge, thus attracting the ferrocyanide ions, since the predicted pKa of 4-ATP is 8.74, conferring a positive charge in the worked 7.14 pH. Corroborating the findings regarding the facilitation in electronic transfer, the results of electrochemical impedance spectroscopy (EIS), presented in Fig S1 and Fig S2, show that the resistance to charge transfer value (R_{CT}) decrease 1.89x and 2.22x when the bare SPCE (1.51 ± 0.03 k Ω) is compared with the SPCE/4-ATP (0.80 ± 0.05 k Ω) and the SPCE/4-ATP/AuNps (0.67 ± 0.05 k Ω), respectively (Supplementary Material).

A reverse behavior was observed in the presence of the cationic probe (Fig. 3b), where the 4-ATP modification decreases the peak current, reinforcing that 4-ATP forms a positively charged layer and repels the positively charged $[Ru(NH_3)_6]^{+2}$ ion. However, Balvedi *et al.* [29], evaluated the behavior of the electrochemical polymerization of the 4-ATP monomer and obtained opposite results to this study, showing that poly(4-ATP) presents anionic behavior comparing interactions with anionic $\{K_3[Fe(CN)_6]/K_4[Fe(CN)_6]\}$ and cationic $\{[Ru(NH_3)_6]Cl_2\}$ probes. This difference is expected since monomeric oxidation and polymeric elongation alters electrostatic interactions between the solution's monomer and in this present work the monolayer rearranges spontaneously in the carbon electrode.

The bare carbon ink working electrode showed a roughness coefficient (R_q) of 138.6 ± 9.2 nm, Fig. 3c – I, and it was possible to see in the SEM images the uncovered carbon flakes of the ink (Fig. 3c – IV). When the electrode was modified with 4-ATP, there was a decrease in the roughness value ($R_q = 112.1 \pm 7.5$ nm, Fig. 3c – II), probably due to the deposition of the monomer in the surface valleys, and the SEM images showed a more homogenous surface (Fig. 3c – V). After the addition of the AuNps modified with the DNA probe there was an increase in the roughness value ($R_q = 147.1 \pm 9.5$ nm, Fig. 3c – III) and it was possible to observe punctual structures peaks with average size of 200 nm, which can be related to the fact that the nanoparticles were adsorbed on the electrode after the drying step. Meanwhile, the SEM images (Fig. 3c – VI) showed a more heterogenous surface in comparison with the carbon ink electrode.

3.2. Functionalization and electrochemical detection of HIV genomic RNA

Ethidium bromide (EB) is a planar aromatic organic compound capable of interacting with the nitrogenous bases of nucleic acids [30]. Detection is possible since EB is electroactive and exhibits different electrochemical behaviors with single-strand and double-strand DNA. Previous studies demonstrated the effect of EB accumulation on the electrode surface when probe and target hybridization occur due to DNA duplex formation, caused by interaction with paired bases. On the other hand, when hybridization does not occur, there is no large accumulation of EB on the electrode surface and the current response is lower [31]. This mechanism is observed using the present bioelectrode by differential pulse voltammetry, shown in Fig. 4a. The BE presented electroactivity on the SPCE/4-ATP/AuNps-HIV1S platform with peak oxidation at approximately + 0.58 V vs. Ag⁺.

The current response change is observed when the complementary

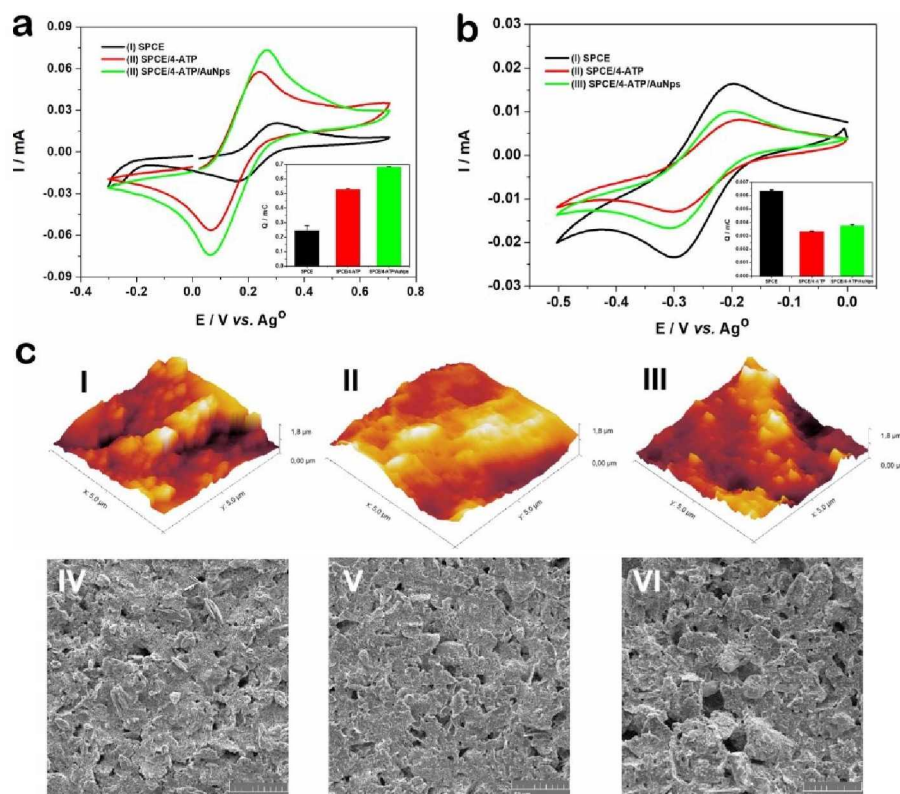


Fig. 3. (a) Cyclic voltammograms with anionic probe: bare SPCE (I), SPCE/4-ATP (II) electrode, and SPCE/4-ATP/AuNps electrode (III). Inset: histograms of the electrical charge involved in the oxidative and reductive processes. Electrolyte: K₃[Fe(CN)₆]/K₄[Fe(CN)₆] (5.0 mmol/L) solution containing KCl (0.10 mol/L), $\nu = 50$ mV/s. (b) Cyclic voltammograms with cationic probe: bare SPCE (I), SPCE/4-ATP electrode (II), and SPCE/4-ATP/AuNps electrode (III). Inset: histograms of the electrical charge involved in the oxidative and reductive processes. Electrolyte: [Ru(NH₃)₆]³⁺/Cl₂ (5.0 mmol/L) solution containing KCl (0.10 mol/L), $\nu = 50$ mV/s. (c) SPM and SEM images of the bare SPCE (I, IV), SPCE/4-ATP (II, V) and SPCE/4-ATP/AuNps + DNA Probe (III, VI).

and non-complementary targets interact with the modified electrode (Fig. 4a). The bare platform with the AuNps-HIV1S probe (Fig. 4a – I) shows the current peak at 4 ± 0.13 μ A and when the HIV viral RNA complementary target (Fig. 4a – II) is added, the current increases by 3.5 times (14 ± 0.3 μ A) and the current response of the non-complementary target of Zika virus viral RNA (Fig. 4a – III) presents the peak current near the probe response at 3 ± 0.2 μ A.

AuNps offer many sites of adsorption for biomolecules, mainly with the use of thiolate probes due to the strong interaction between gold and thiol groups [32]. The large surface area of the AuNps also provides an efficient immobilization of biomolecules, which facilitates the detection of the target analytes [22]. The EB is a hydrophobic molecule with affinity for DNA-RNA double-strands, composed of its hydrophobic nitrogen bases in the hydrophilic aqueous environment of plasma, but not by single-strand of DNA or RNA [30]. The smallest signal of electrical current of the probe and the non-complementary target is expected in relation to the complementary target, resulting from the intercalation of EB to the double-strands formed. Thus, the platform SPCE/4-ATP/AuNps-HIV1S proved to be selective in the discrimination between positive and negative target in a complex sample of human plasma.

Scanning probe microscopy can be a valuable technique to evaluate

the immobilization and hybridization of DNA and RNA in solid surfaces [33]. The addition of the negative RNA target (ZIKV RNA) did not significantly alter the roughness coefficient ($R_q = 139.3 \pm 10.2$ nm, Fig. 4b – I) in relation with the probe (Fig. 3c – III). However, the addition of the positive target (HIV RNA) increased the roughness coefficient ($R_q = 201.6 \pm 15.9$ nm, Fig. 4b – II) and it was possible to identify globular structures with average size of 500 nm. Double stranded nucleic acids are less flexible than single stranded nucleic acids [34], thus the hybridization of the DNA oligonucleotide present on the electrode surface with the complementary RNA present in the sample creates more prominent clusters on the electrode surface [35]. It was also possible to observe different morphologies in the SEM images, with the addition of the positive target (Fig. 4b – IV) caused a change in the surface of the working electrode, making it more heterogeneous in comparison with the negative target (Fig. 4b – III). This may be due aggregation of the nanoparticles after adding the positive target.

Analytical responses by differential pulse voltammetry with different concentrations of HIV gRNA are shown in Fig. 5a versus the variation of the gRNA concentration logarithm in copies per mL (Cp/mL). Fig. 5b shows the current response versus the potential oxidation window of ethidium bromide. The order is from lowest concentration to highest

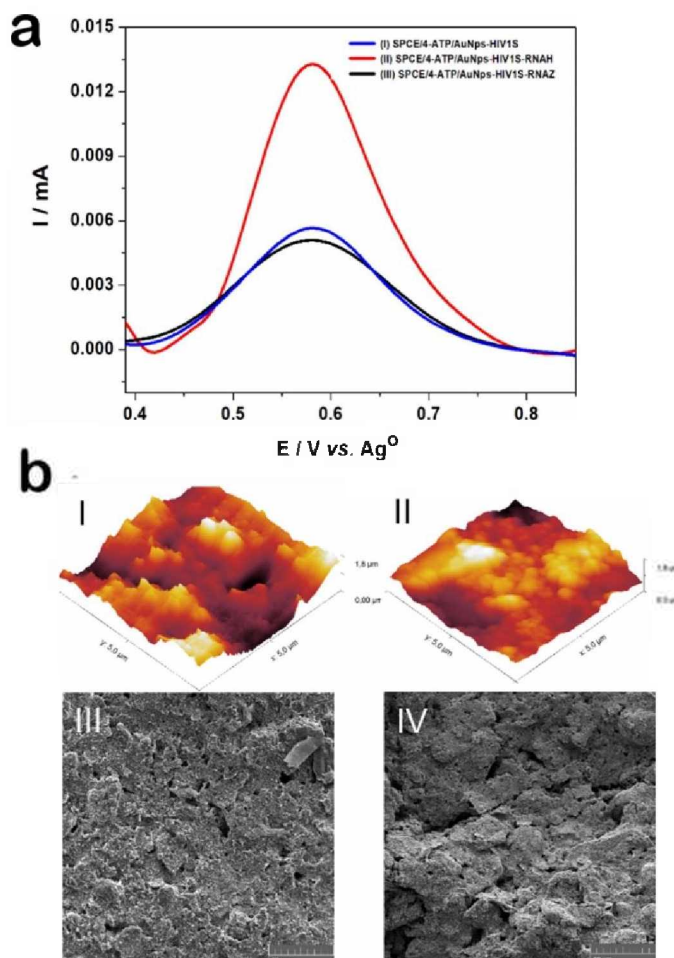


Fig. 4. (a) Differential pulse voltammograms of screen-printed carbon electrode (SPCE) modified with 4-ATP containing AuNps-HIV1S in the presence of ethidium bromide before (I) and after interaction with the complementary target (HIV-RNA, II) and non-complementary target (ZIKA-RNA, III). Electrolyte: phosphate buffer (0.10 mol/L), pH 7.4, amplitude 0.07 V, pulse interval, 0.2 s, $v = 30$ mV/s. (b) SPM and SEM images of WE/4-ATP/AuNps + DNA Probe/ZIKA RNA (I, III) and WE/4-ATP/AuNps + DNA Probe/HIV RNA (II, IV).

concentration of the $\times$ axis: 6 ($5.33 \pm 0.40 \mu\text{A}$), 56 ($7.85 \pm 0.63 \mu\text{A}$), 560 ($11.00 \pm 0.60 \mu\text{A}$), 1100 ($12.00 \pm 0.46 \mu\text{A}$) and 11,000 ($14.60 \pm 0.29 \mu\text{A}$) Cp/mL, diluted 1:10000, 1:1000, 1:100, 1:50 and 1:5 in plasma, respectively. The calibration curve of the genosensor was constructed, as the oxidation peak current of the EB indicator correlates with the logarithm of the concentration of genomic RNA of HIV. The experimental calibration curve can be fitted by a linear straight line of equation $ip(\mu\text{A}) = 2.91(\mu\text{A/Cp/mL}) \cdot \log[\text{gRNA}](\text{Cp/mL}) + 3.04(\mu\text{A})$, $R^2 = 0.9989$ ($N = 3$). There was a linear response range between 6 Cp/mL (6.5 ng/mL) to 11,000 Cp/mL (13 $\mu\text{g/mL}$). The biosensor detected up to 6 Cp/mL. This value is much lower when compared to commercial RT-PCR-based that detect up to 20, 30 and 40 Cp/mL [36].

Plasma samples doped with HIV genomic RNA (length of ~ 10 kb) [37] were used to validate the biosensor. Fig. 5c shows the response of 6 independent samples compared to the probe and two negative samples, one of Zika virus genomic RNA and another of genomic DNA of *S. aureus* bacteria. All samples differed from the probe and negative, showing the

specificity and selectivity of the HIV1S probe with different genetic materials in a complex protein matrix, an intrinsic characteristic of human plasma. The recently developed platforms (Table 1) exhibit remarkable analytical parameters in biosensing, however, most use synthetic sequences of complementary DNA to validate the biosensor and not the complex biomolecule of HIV RNA. This can be a major challenge with regard to the possibility of transforming technology at the level of research for commercial applications. This work uses the native RNA molecule in its environment, allowing it to be in its usual conformation. In addition, it is important to note that, as mentioned in item 2.6, different electrodes with the same electrochemical profiles were selected for all studies of this work. This demonstrates that our artisanal electrodes have high reproducibility for future commercial applications.

The concentrations of the positive samples were determined using the calibration curve data (Fig. 5c): (A1) 41.67 Cp/mL; (A2) 354.81 50 Cp/mL; (A3) 15.49 Cp/mL; (A4) 10.42 Cp/mL; (A5) 2000 Cp/mL; (A6)

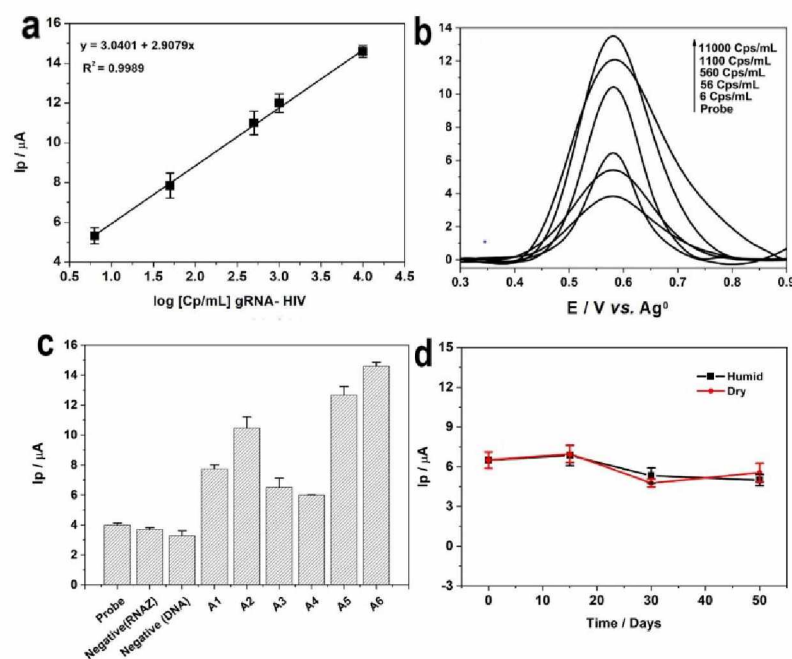


Fig. 5. (a) Calibration curve of the biosensor. (b) Differential pulse voltammograms in phosphate buffer. (c) Histogram showing the peak current (I_p) for positive: gRNA HIV-affected individuals (A1-A6) and negative samples of Zika virus RNA (RNAZ) and *Staphylococcus aureus* (DNA). (d) Storage stability in a humid and dry inert environment. Electrolyte: phosphate buffer (0.10 mol/L), pH 7.4. Modulation amplitude 70 mV, pulse interval 0.2 s; $v = 30 \text{ mV/s}$; $N = 3$.

Table 1

Recent examples of electrochemical biosensor for HIV detection.

Electrode	Platform	Technique	Detection limit	DLR	Stability	Sample	Sample Volume	Target	Reference
Commercial SPCE	Graphene-Nafion	CV/DPV	5 nM	40 nM to 2.56 μM	30 days	Serum	50 μL	Synthetic DNA oligonucleotides	[39]
GCE	Graphene-Nafion	EIS	$2.3 \times 10^{-14} \text{ M}$	1.0×10^{-13} to $1.0 \times 10^{-10} \text{ M}$	1 week	Buffer	5 μL	Synthetic DNA oligonucleotides	[40]
GCE	Molecularly imprinted polymers/MWCNTs	DPV	0.083 $\mu\text{g cm}^{-2}$	1.0×10^{-4} to 2 ng cm^{-2}	5 days	Serum	5 μL	Protein	[41]
CPE	Ion-imprinted polymer nanoparticles/AuNps	DPV	0.3 fM	1 fM to 0.3 fM	Not reported	Serum	5 μL	Synthetic DNA oligonucleotides	[42]
Au electrode	—	DPV	0.21 pM	1 pM to 100 nM	Not reported	Serum	100 μL	Synthetic DNA oligonucleotides	[43]
GCE	Amino reduced graphene oxide/ β -cyclodextrin	DPV	8.7 fM	0.05 pM to 1 pM	1 week	Serum	5 μL	Synthetic DNA oligonucleotides	[44]
Artisinal SPCE	4-aminophenol/AuNps	DPV	6 Cp/mL (6.5 ng)	6 to 11,000 Cp/mL (6.5 ng to 13 μg)	15–30 days	Plasma	3–5 μL	Viral Genomic RNA	This work.

10,232 Cp/mL. The current varying linearly with the viral load indicates that in addition to the qualitative possibility of detection, it is also possible the quantitative detection of the viral load using this methodology in this response range. This is a crucial factor for the development of biosensors for the early detection of HIV, since it is only possible to detect the genetic material in the first days of infection and in very low concentrations. Thus, these results indicate an important step towards the development of quantitative and qualitative point-of-care diagnostics.

Recent electrochemical biosensors (Table 1) share good performance regarding the detection limit but have gaps in the detection of real viral

RNA, since most detect synthetic DNA molecules. This work shows the possibility of detecting HIV viral RNA in a stable, sensitive and accessible manner. It is important to highlight that the present biosensor detects the range considered clinically relevant, between 50 and 200 Cp/mL, because it is related to resistance to antiretroviral therapy and is the focus of continuous monitoring in HIV-infected patients [36].

Stability is an important parameter for future commercial applications of new biosensor platforms [45]. Carbon-based materials are inert due to the hydrophobic nature of the carbon network and disperse polar solvents poorly [46]. The functionalization of these platforms can influence both the change in the reactivity of carbon ink and the

conformation of biomolecules inserted for biorecognition.

In the present work, to analyze the humidity factor in improving or worsening stability, the electrodes were stored in an inert atmosphere (N_2) with or without humidity in the environment (Fig. 5d). All the ready-to-use genosensors (SPCE/4-ATP/AuNps-HIVIS) were stored under the same conditions. For each evaluated day, DPV was performed to detect HIV RNA (target) in triplicate. The same target sample was used both on day 1 and on the rest of the days for comparison. The electrodes stored in a wet or dry chamber presented the same original response after 15 days. The current response maintained in a humid environment, on thirtieth and fiftieth day, the response dropped by 81.5% and 77%, respectively, and 73.5% and 85.5%, in the electrodes without humidity, respectively. Therefore, it is reasonable to infer that humidity influences the platform response up to 15 days of storage. Then, the response drops progressively in the humid environment and within 50 days there was a response's increase in the dry environment, probably due to operational problems, such as errors in pipetting and storage. The use of wet chamber has already been reported in previous work [47]. Hence, the use of dry storage helps in the reduction of biosensor's production stages. According to Table 1, our artisanal electrode is promising since stability is better or similar to new platforms for detecting HIV genetic materials using commercial electrodes. The reproducibility of our artisanal electrodes is 90%, that is, for every 10 electrodes manufactured, 9 have the same electrochemical profile.

4. Conclusions

Electrochemical biosensors are rapid, cheap and portable alternatives to conventional methods for detecting HIV biomarkers. There are also gaps in HIV diagnosis in the initial period of the disease, called acute infection stage. At this stage it is only possible to detect genetic materials of the virus and the p24 antigen and in very low amounts that, consequently, only sensitive methods achieve.

Here we present a new methodology for SPCE construction in a simple way, without many stages for production, using carbon ink and vinyl adhesives. The simplicity of the electrodes construction for biosensing is an important factor for future commercial applications, especially in areas with limited resources for HIV diagnosis.

The SPCEs were modified with a 4-ATP monolayer which improved the electronic properties of the carbon-based platform, besides constituting an anchoring network for gold nanoparticles functionalized with thiolate probe. The cationic characteristic of the monolayer was observed with its characterization using anionic redox probe $[K_3[Fe(CN)_6]/K_4[Fe(CN)_6]]$ and cationic probe $[Ru(NH_3)_6]Cl_2$. In addition, studies with SEM and SPM showed the roughness change with the use of 4-ATP and AuNps decorated with a thiolate probe specific to HIV.

EB is an efficient DNA intercalant and indicates the occurrence or not of hybridization due to its ability to bind to double-strand, but not to the single-strand of DNA. EB electroactivity was observed in +0.58 V and the current response decreases when comparing the presence or absence of HIV RNA. The SPCE/4-ATP/AuNps-HIVIS bioelectrode was validated with real viral RNA samples enriched with blood plasma and non-complementary targets of RNA (Zika virus) and DNA (*S. aureus*), showing specificity and selectivity. The calibration curve was constructed and the current response of EB was linear related to the logarithm of concentration of HIV RNA, detected up to 6 Cp/mL and linear range between 6 and 11,000 copies of RNA, covering the clinically relevant concentration range (50–200 Cp/mL) for monitoring antiretroviral treatment, detecting extremely low concentrations of the virus, an acute phase infections characteristic. The stability of the genosensor was analysed in a dry and humid environment and it remained stable for 15 days in both conditions.

Furthermore, this work shows the possibility of simple construction of electrodes with commercial applications in point-of-care diagnosis for the detection of viral RNA, presenting sensitivity, selectivity, specificity and stability.

CRedit authorship contribution statement

Anna C.R. Moço: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft. João A.S. Neto: Conceptualization, Methodology. Dayane D. Moraes: Methodology, Investigation, Validation. Pedro H. Guedes: Methodology, Investigation, Validation. Jéssica G. Brussasco: Methodology, Investigation, Validation. José M.R. Flauzino: Methodology, Validation, Formal analysis. Luiz F.G. Luz: Validation, Formal analysis. Márcia M.C.N. Soares: Validation, Formal analysis. João M. Madurro: Supervision, Writing - review & editing, Formal analysis. Ana G. Brito-Madurro: Supervision, Writing - review & editing, Formal analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.microc.2021.106739>.

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