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ESTUDO DA INTERAÇÃO DE ÁCIDOS NUCLEICOS COM O NANOPORO ADAPTADO DA α-HEMOLISINA

ANNIELLE MENDES BRITO DA SILVA

Orientadora: Professora Doutora Adriana Fontes
Co-orientador: Professor Doutor Gustavo de Miranda Seabra

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Dissertação de Mestrado apresentada ao Programa de Pós-Graduação em Ciências Biológicas da Universidade Federal de Pernambuco, como parte dos requisitos para obtenção do título de Mestre em Ciências na área de Concentração em Biotecnologia.

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“Bem-aventurado o homem que acha sabedoria, e o homem que adquire conhecimento.” (Provérbios 3:13)

"*Faça o que puder, com aquilo que você tem, onde você está.*" (*Theodore Roosevelt)*

“Nós somos madeira de lei que cupim não rói.” (Capiba)

RESUMO

O nanoporo formado pela incorporação da α-hemolisina em bicamadas lipídicas planas é considerado modelo de nanoporo proteico para elucidação do mecanismo de transporte de moléculas e no desenvolvimento de dispositivos analíticos - biossensores, espectrômetros de massa e sequenciadores moleculares. O conhecimento da interação de nucleotídeos com o nanoporo da α-hemolisina é de especial interesse, pois, alguns estudos sugerem varias metodologias para a utilização deste nanoporo como sequenciador de DNA em tempo real. Apesar de todos os avanços, a principal dificuldade operacional para obtenção de um sequenciador baseado na tecnologia "nanopore sensing", é a rapidez na translocação do DNA através do nanoporo; dificultando a discriminação adequada das bases. Neste contexto é imprescindível fazer adaptações moleculares no nanoporo visando o aumento do tempo de permanência do DNA e da energia de interação deste com o nanoporo. As principais estratégias disponíveis para produção de nanoporos adaptados são: mutações sítio dirigidas e funcionalização química. Ambas são de elevado custo e tempo de experimentação. Neste trabalho utilizamos técnicas de simulação computacional para obtenção, a nível atomístico, a interação do DNA com o nanoporo da α-hemolisina na sua forma nativa e adaptada em posições estratégicas previamente selecionadas por modelagem molecular. As técnicas utilizadas baseiam-se na dinâmica molecular fora do equilíbrio e na Relação de Jarzynski, na qual a média do trabalho realizado ao deslocar o DNA ao longo do nanoporo proteico é estatisticamente relacionada à energia livre do processo. As informações sobre as interações do DNA-nanoporo obtidas podem predizer, teoricamente, os nanoporos mais promissores para serem testados experimentalmente. Realizou-se a seleção das mutantes que foram usadas e foram obtidos dados importantes sobre a parametrização das dinâmicas usando a relação de Jarzynski, como velocidade e constante de força que devem ser aplicadas ao sistema. Além disso, foram obtidas informações sobre a trajetória e contato do DNA com o interior do poro mutado e na forma selvagem, o que mostra a efetividade do sistema.

Palavras-chave: α-hemolisina, nanoporo, ácidos nucleicos, dinâmica molecular, energia livre, método de Jarzynski.

ABSTRACT

The nanopore formed by incorporation of α-hemolysin in planar lipid bilayers is considered a model protein nanopore for studying the mechanism of molecular transport and the development of analytical devices - biosensors, mass spectrometers and molecular sequencers. The understanding of the nucleotides interaction with the α-hemolysin nanopore is of special interest because some studies suggest several methods for using this as nanopore DNA sequencer in real time. Despite all the advances, the main operational difficulty to obtain a sequencer based on "nanopore sensing" technology is the fast translocation speed of the DNA through the nanopore, which hinders proper discrimination of the bases. In this context it is essential to make molecular adaptations to the nanopore in order to increase the residence time of the DNA and the interaction energy with the nanopore. The main strategies available for producing tailored nanopores are: site directed mutations and chemical functionalization. Both are costly and demand much experimentation. In this work we use computer simulation techniques to obtain, on the atomistic level, the interaction of DNA with α-hemolysin nanopores in its native form and adapted in strategic positions previously selected by molecular modeling investigations. The techniques used are based on nonequilibrium molecular dynamics and the Jarzynsnki's relation, wherein the average of the work performed by shifting the DNA along the nanopore protein is statistically related to the free energy of the process. We will use the obtained information about the interactions of DNA-nanopore to theoretically predict the most promising nanopores to be tested experimentally. We presented here a new model system for the simulation of DNA translocation through the α-hemolysin pore. The pore was constructed in vacuum, and restraints and implicit solvent with a low dielectric constant used to simulate the membrane environment. The control parameters for the multiple steered molecular dynamics simulations needed to obtain the potentials of mean force of translocation, pulling speed and force constant, were determined and tested with simulations of DNA translocation using the wild type and two different mutant pores, which were characterized by their translocation PMF, hydrogen bonding pattern, and pore diameter variation through the simulations. The results are promising and show that the model is stable and respond adequately to the changes in the pore.

Key-words: α-hemolysin, nanopore, nucleic acids, molecular dynamics, free energy, Jarzynski's method.

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1. INTRODUÇÃO

O desejo de sequenciar o genoma humano rapidamente e por um valor acessível é um ponto de extrema importância na medicina, e de grande interesse comercial e tecnológico. A esperada acessibilidade e velocidade seria possível, por exemplo, ter o genoma sequenciado para um tratamento médico personalizado (WHEELER et al., 2008). Graças aos avanços da segunda geração de técnicas de sequenciamento de DNA, na primeira década do século XXI, o custo do sequenciamento de um genoma humano inteiro era cerca de U.S. $50.000 (BONETTA, 2010). No entanto, as tecnologias de segunda geração como 454 Life Sciences (Roche), Solexa (Ilumina) e Applied Biosystems SOLiD (Life Technologies), dependem de lentos ciclos de processamento enzimático e coleta de dados baseada em imagens (SHENDURE & JI, 2008). Neste contexto os candidatos mais prováveis para diminuição do custo e o tempo do sequenciamento do genoma humano seriam os sequenciadores de terceira geração, como o Pacific Bioscience's, sequenciamento de moléculas simples em tempo real por síntese (SMRT), baseado na determinação contínua de sequências de moléculas individuais de DNA por processos isentos de ciclos. Outra alternativa, mais viável, seria o sequenciamento por nanoporo, que também faz parte da terceira geração de sequenciadores (WANUNU, 2012).

Na abordagem do sequenciamento por nanoporo (tecnologia "*nanopore sensing*"), uma fita simples de molécula de DNA ou RNA é conduzida eletroforeticamente através de um único nanoporo e cada base é lida à medida que passa em um ponto de reconhecimento no interior do nanoporo. A corrente que é gerada pela passagem de íons (por exemplo, K^+ e Cl^-) através do nanoporo durante a translocação do DNA proporciona um sinal elétrico necessário para distinção de cada base. O nanoporo proteico formado pela α-hemolisina, proteína produzida pela bactéria *Staphylococccus aureus*, quando incorporado em uma bicamada lipídica plana, é considerado como uma das mais promissoras nanobioestruturas na produção de sequenciadores moleculares, uma vez que já se elucidou sua estrutura molecular cristalina (SONG et al., 1996); possui dimensão geométrica compatível com o DNA; e facilidade de adaptação por meio técnicas de engenharia genética e/ou modificação química (CLARKE et al., 2009; AKSIMENTIEV, 2010).

Um dos maiores problemas para utilização dos nanoporos de α-hemolisina como sequenciadores de ácidos nucleicos é a alta velocidade de translocação da fita de DNA através do interior do nanoporo. Isso faz com que a detecção das bases não ocorra de modo

eficaz e com confiabilidade. Neste contexto, buscam-se modos para diminuir a velocidade de translocação da fita de DNA e, consequentemente, aumentar o tempo de residência deste no interior do nanoporo proteico. As estratégias para superação desta dificuldade operacional consistem em: alteração na concentração ou composição das soluções iônicas (RODRIGUES et al., 2008; RODRIGUES et al., 2011; KOWALCZYK et al., 2012); ou alterações na estrutura da própria proteína, através de mutações sítio-dirigidas e/ou funcionalização química (MERZLYAK et al., 2005; CLARKE et al., 2009; RINCON-RESTREPO et al., 2011).

Neste trabalho empregamos técnicas de dinâmica molecular, usando softwares apropriados para os cálculos e análises. Desta forma, temos uma ferramenta para avaliar a modificação estrutural do nanoporo de α-hemolisina, analisando o perfil de energia livre, e assim obtendo sua interação com ácidos nucleicos, visando à predição de qual nanoporo modificado seria mais apto para aplicação como sequenciador de DNA.

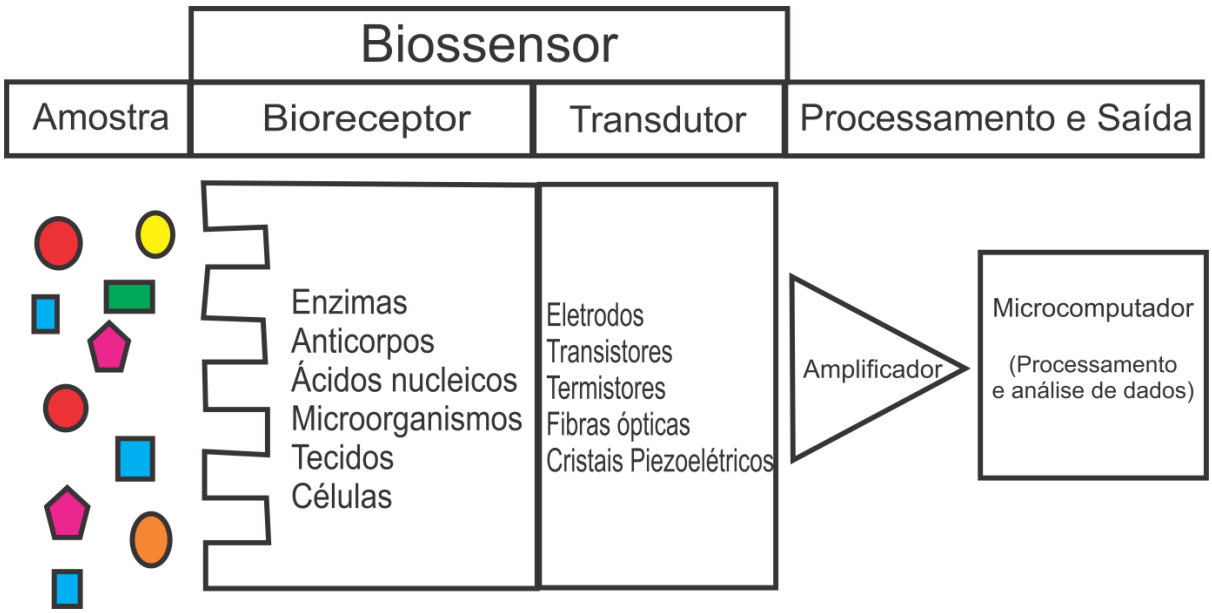
2. REVISÃO DA LITERATURA

2.1.BIOSSENSORES: APLICAÇÕES E CLASSIFICAÇÃO

O biossensor é um dispositivo analítico capaz de detectar moléculas com características individuais (analito), além de permitir a aquisição, leitura ou transmissão de uma informação, no qual o elemento sensor é um material biológico ou derivado dele (ex: uma enzima, um anticorpo, uma proteína, DNA, etc.) (LOWE, 2007; VELUSAMY et al, 2010).

Este dispositivo pode ser representado esquematicamente por três partes **(Figura 1)**. A primeira (camada de reconhecimento biológico) é responsável pela detecção e é formada por uma molécula com suas funções biológicas ativas. A segunda parte é um transdutor de sinais que funciona como um estágio que interpreta os eventos biológicos, ocorridos na etapa de detecção, e os transforma em sinais eletrônicos. A terceira parte (processamento) diz respeito à interface onde são processados os sinais advindos do transdutor para torná-los visíveis em uma interface qualquer, como um medidor, um display ou monitor de computador (VELUSAMY et al.,2010).

Figura 1. Esquema geral dos componentes de um biossensor. O analito (amostra) entra em contato com a camada e reconhecimento (bioreceptor), este contato gera um sinal que é levado ao transdutor, posteriormente, amplificado e processado para análise (normalmente em microcomputador).



2.1.1 Aplicações dos Biossensores

Nos últimos anos, houve um aumento no interesse em pesquisas com biossensores devido à sua ampla aplicação em diversas áreas (LUONG et al., 2008; CLOAREC et al. 2008). No diagnóstico clínico e monitoramento da saúde tem-se o principal campo de pesquisa na área de biossensores, e está relacionado com a melhoria da qualidade de vida de pacientes, sendo realizado com rapidez e precisão (D'ORAZIO, 2011). Por muitas vezes os biossensores mostram resultados em tempo real. O exemplo que possui maior aplicabilidade, são os aparelhos portáteis para monitoramento da glicemia, principalmente usados por pacientes diabéticos. Estes aparelhos são "eletrodos amperimétricos enzimáticos", baseando-se na atividade da enzima glicose oxidase (FERNANDES, 2005; WANG, 2008). Também foram desenvolvidos sensores para a detecção de agentes patogênicos (NAYAK et al., 2009). Recentemente vem sendo desenvolvido um biossensor para a detecção de novos compostos com atividade anticancerígena (ABOU-GHARBIA et al., 2012).

Na indústria alimentícia, os biossensores são usados para controlar os processos de elaboração e seleção dos alimentos de modo a detectar alimentos contaminados , que podem afetar a saúde (FURTADO et al., 2008; VELUSAMY et al., 2010). No controle de agentes poluentes e contaminantes do meio ambiente, permitindo a medição portátil ou monitoramento contínuo, sendo assim bastante útil para medir a contaminação do meio ambiente por determinadas substâncias, principalmente, no monitoramento da presença de gases tóxicos nas proximidades de indústrias, ou mesmo ambientes suscetíveis a grandes cargas de poluição urbana (WANG et al., 2009) e contaminação por inseticidas (ALONSO et al., 2012).

Adicionalmente também estão sendo realizadas pesquisas voltadas para a área militar, na detecção de agentes tóxicos que podem der usados como armas biológicas (POHANKA et al., 2008; POHANKA et al., 2013).

2.1.2 Classificação dos Biossensores

Os biossensores podem ser classificados levando em consideração aspectos do mecanismo de detecção, tipo de transdução e também tipo de interação entre o analito e o elemento de reconhecimento (camada de reconhecimento biológico).

Primeiramente podemos classificar os biossensores quanto à natureza do elemento sensor ou de reconhecimento:

I. Biossensores baseados em sistemas vivos, cuja resposta se deve ao funcionamento de um órgão ou organismo inteiro. Podemos citar órgãos olfatórios como antenas de crustáceos e de baratas, e até o nariz humano (ZENG WU, 1999); ou organismos inteiros, como por exemplo o que acontece em resposta à contaminação de rios e mananciais, pois alguns organismos respondem em concentrações muito mais baixas do que os seres humanos podem detectar para alertar sobre a presença de metais pesados e toxinas. (KAHRU et al., 2005);

II. Biossensores baseados em células íntegras, cuja operação se deve ao funcionamento do conjunto de células ou a uma única célula dissociada (QU et al., 2013; HAHN & TOUTCHKINE, 2002);

III. Biossensores baseados em enzimas, que funcionam em resposta a ação de uma enzima sobre seu substrato. O exemplo clássico é o acoplamento de uma enzima ao eletrodo de oxigênio de Clarke (GRIESHABER et al., 2007); Como citado anteriormente, o biossensor para a glicemia é baseado na atividade da enzima glicose oxidase, que é uma proteína dimérica que catalisa a oxidação da beta-D-glicose em D-glucono-delta-1,5-lactona, que depois é hidrolisada em ácido glucurônico. Esta enzima é usada no biossensor para detecção de níveis de glicose. Isto é efetuado através do registro do número de elétrons que passam pela enzima que é conectada a um eletrodo medindo a carga resultante. Desta forma obtemos a concentração de glicose no visor do aparelho (LI & YUAN, 2006);

IV. Biossensores baseados em anticorpos, cujo funcionamento resulta da reação entre moléculas do sistema imune e antígenos. Podemos citar os ensaios imunoenzimáticos e o radioimunoensaio, são os chamados imunossensores (WANG et al., 2013; SHANKARAN et al., 2007**)**;

V. Biossensores baseados em receptores de membrana, funcionando em resposta a interação de um único receptor ou grupo de receptores com seus ligantes. Citamos

principalmente os ensaios usando a técnica de patch-clamp, em sistemas comerciais robotizados lançados no mercado (ERAY et al., 1995)

VI. Biossensores baseados em moléculas de DNA imobilizadas, chamados de Genossensores. Estes dispositivos podem monitorar processos de hibridização de sequências específicas por meio de detecção direta das bases mais eletroativas do DNA (guanina e adenina), ou pela detecção indireta da hibridização, por meio de indicadores, marcadores enzimáticos, marcadores de nanopartículas ou pelo uso de intercaladores de DNA que formam complexos com suas bases nitrogenadas, estes dispositivos podem detectar sequencias de DNA específicas de determinados agentes causadores de patologias (KERMAN et al., 2004; PRABHAKAR et al., 2008).

De acordo com o transdutor utilizado, os biossensores podem ser classificado como (PATACAS, 2007):

I. Eletroquímico: Os biossensores eletroquímicos podem ser de três tipos: amperométrico, condutimétrico ou potenciométrico (THÉVENOT et al., 2001). Os biossensores amperométricos são baseados na medida de corrente elétrica resultante de alterações de oxidação ou redução de espécies eletroativas. Os biossensores potenciométricos se baseiam na diferença de potencial entre dois eletrodos em condições de corrente elétrica constante; enquanto nos biossensores condutimétricos as mudanças são observadas nas medidas de condutância, resultante de produtos de reação catalítica (KERMAN et al., 2004; GRIESHABER et al, 2007).

II. Óptico: Biossensores ópticos baseiam-se na determinação das mudanças de absorção de radiação eletromagnética na região do visível/infravermelho entre os reagentes e produtos da reação, ou na medida da emissão de luz por um processo luminescente. Podem ser baseados na medida de luminescência, fluorescência, etc. (TELES & FONSECA, 2008).

III. Detector de massa ou Piezoelétrico: Detecta alteração de massa e/ou microviscosidade. Piezoeletricidade é a propriedade dos cristais de gerar uma tensão em resposta a uma vibração externa. Este efeito é reversível e, dessa forma, todos os cristais piezoéletricos vibram na presença de um campo elétrico. A freqüência (f) dessa vibração depende da espessura e do corte do cristal, sendo que cada cristal possui uma freqüência de vibração característica. Essa freqüência característica muda quando o cristal adsorve ou desorve moléculas em sua superfície. Como a variação de freqüência é proporcional à

variação de massa do material adsorvido, tal variação pode ser determinada por circuitos eletrônicos através da medida de impedância (SANG-MOK et al., 2000).

IV. Termométrico: Baseia-se na propriedade fundamental das reações químicas, ou seja, absorção ou emissão de calor. A quantidade de calor liberado ou absorvido durante a reação é proporcional ao número total de moléculas do produto formado na reação (RAMANATHAN et al., 2001).

A depender do tipo de interação que ocorre entre a substância a ser detectada (analito) e o material biológico, o biossensor pode ser classificado como catalítico ou por afinidade (RICCARDI et al, 2002; FURTADO et al., 2008). No catalítico há perda de material, ou transformação do analito inicial em um produto. No biossensor por afinidade temos interações físicas entre o analito e o elemento sensor, mas o analito permanece com suas características iniciais.

O biossensor abordado neste trabalho é baseado no poro unitário formado pela α-hemolisina de *Staphylococcus aureus* inserido em uma bicamada lipídica plana, sendo este dispositivo uma nova classe de sensores, denominados estocásticos (BAYLEY & CREMER, 2001; MOVILEANU, 2009; RODRIGUES, 2006).

2.2.NANOPORO DE α-HEMOLISINA COMO BIOSSENSOR ESTOCÁSTICO

A α-hemolisina, secretada pela bactéria *Staphylococcus aureus*, é uma citolisina dotada da capacidade de formar poros aquosos tanto em membranas biológicas quanto em bicamadas lipídicas artificiais (KRASILNIKOV et al, 2000). A proteína possui peso molecular em torno de 33,2 kDa e, em solução aquosa, encontra-se na forma de monômeros polipeptídicos de 293 aminoácidos (GRAY & KEHOE, 1984), os quais oligomerizam-se e se inserem na membrana, formando um poro heptamérico transmembranar que permite a passagem de água e íons e a interação de diversas moléculas com o seu interior (GU et al, 1999; KRASILNIKOV et al, 2000; NOSKOV et al, 2004).

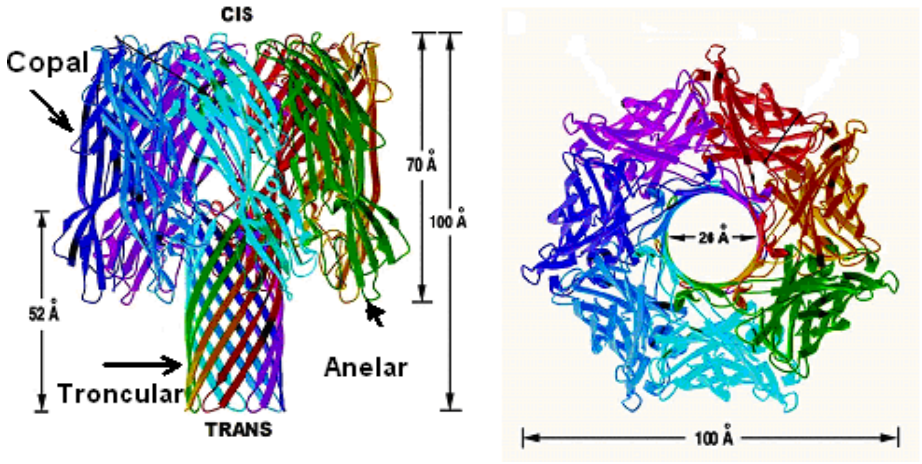
A estrutura do heptâmero detergente-solubilizado foi determinada por cristalografia de raio-X em 1,9Å de resolução. A estrutura cristalográfica foi depositada no *Protein Data Bank* (RCBS-PDB) como 7AHL (SONG et al.,1996). Com a forma semelhante à de um cogumelo, o poro heptamérico é caracterizado por apresentar três domínios: copal, anelar e troncular. O nanoporo mede aproximadamente 10 nm de altura e 10 nm de diâmetro externo. O eixo central chamado de lúmen, por onde há a passagem de água e solutos, possui uma constrição com diâmetro aproximado de 1,2 nm, esta constrição localiza-se na região entre o tronco e a estrutura copal, mais especificamente na posição 147, onde há um aminoácido lisina (GOUAUX, 1998). O restante do poro interno varia seu diâmetro de 1,4 a 4,6 nm, sendo que no domínio troncular o diâmetro é de ~2,6 nm, este é composto por 14 folhas β-barril antiparalelas e corresponde à parte que efetivamente está inserida na membrana celular. O domínio copal, com diâmetro de 4,6 nm, projeta-se na superfície extracelular e forma uma larga extensão hidrofílica (SONG et al.,1996). O domínio anelar encontra-se na parte inferior do copal e, provavelmente, interage com a superfície da membrana. Estas características do nanoporo permitem o seu uso como principal elemento de reconhecimento na construção de um biossensor para a detecção de diversas substâncias em meio aquoso (BAYLEY & CREMER, 2001; NOSKOV et al, 2004). A estrutura do nanoporo com detalhes de suas dimensões estão mostrados na **Figura 2**.

O nanoporo proteico é útil na criação de sensores multianalitos com inúmeras aplicações. Estudos mostram que os nanoporos unitários de α-hemolisina podem ser usados para detectar e quantificar íons (mono- e divalentes) (KASIANOWICZ et al., 1999), moléculas orgânicas pequenas (BEZRUKOV et al., 2004; KANG et al., 2006), proteínas e peptídeos (WOLFE et al., 2007; ZHAO et al., 2009), moléculas poliméricas (KRASILNIKOV et al., 2006; RODRIGUES et al., 2008, RODRIGUES et al., 2011), ácidos nucleicos

(CLARKE et al., 2009; AKSIMENTIEV, 2010; TIMP et al., 2010; WANUNU, 2012), e compostos nocivos (WU & BAYLEY, 2008; LIU et al., 2010).

O princípio do sensor estocástico baseia-se na detecção feita pelo nanoporo unitário para identificar e quantificar diversas moléculas em solução aquosa (MARTIN & SIWY, 2007; MOVILEANU, 2008). As moléculas do analito se aproximam do nanoporo por difusão e interagem temporariamente com o interior do nanoporo. O resultado de cada interação se reflete na mudança transitória (evento) da corrente iônica que passa através do nanoporo **(Figura 3)**. A distribuição desses eventos na escala do tempo é aleatória, permitindo a aplicação da terminologia, detecção estocástica ou sensor estocástico (MOVILEANU, 2009).

Figura 2. Modelo do nanoporo formado pela α-hemolisina com especificações de suas dimensões e domínios (anelar, copal e troncular), com visão lateral e frontal da região troncular. De um modo geral, a região troncular fica inserida na membrana, a região copal prolonga-se para a superfície formando um canal hidrofílico e a região anelar recobre a parte inferior da região copal podendo ter alguma interação com a membrana. À direita, temos uma visão a partir do lado TRANS, onde se mostra a medida do diâmetro aproximado do poro na região troncular (~26 Å). Adaptado de SONG et al., 1996.



O biossensor estocástico é normalmente construído pela incorporação de um único nanoporo em uma bicamada lipídica que separa dois compartimentos contendo soluções aquosas **(Figura 4)**. O nanoporo é a única via de condução elétrica entre os dois compartimentos. Um potencial elétrico aplicado entre os dois compartimentos, ou seja, transmembrana, faz com que os íons gerem uma corrente iônica que flui pelo nanoporo. (AKSIMENTIEV, 2010; CHEN & CONLISK, 2010). Quando um analito de dimensões compatíveis com a do nanoporo; adicionado a um dos compartimentos entra e/ou permeia o nanoporo, gera uma mudança (evento) na corrente iônica. A frequência de ocorrência dos eventos está diretamente relacionada com a concentração, enquanto que a "assinatura digital de corrente", ou seja, a série temporal dos eventos (tempo característico de duração e a profundidade de bloqueio) permitem a identificação do analito (Figura 3).

Figura 3. Na ausência de qualquer molécula, a corrente iônica que passa pelo canal é máxima e estável, quando há um potencial aplicado. Por difusão, a molécula chega ao interior do canal e interage com o poro modulando a sua corrente de maneira característica dependendo das propriedades da molécula. Estas características são dadas pelo tempo de permanência e profundidade de bloqueio. As setas azuis indicam o sentido de movimentação do analito.

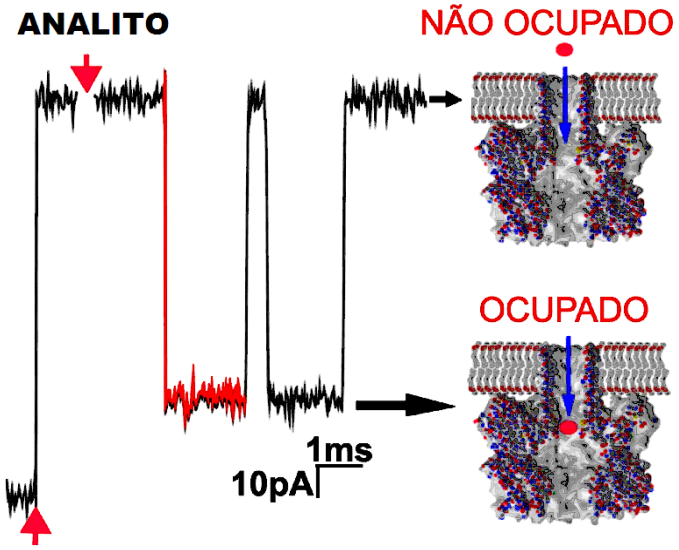
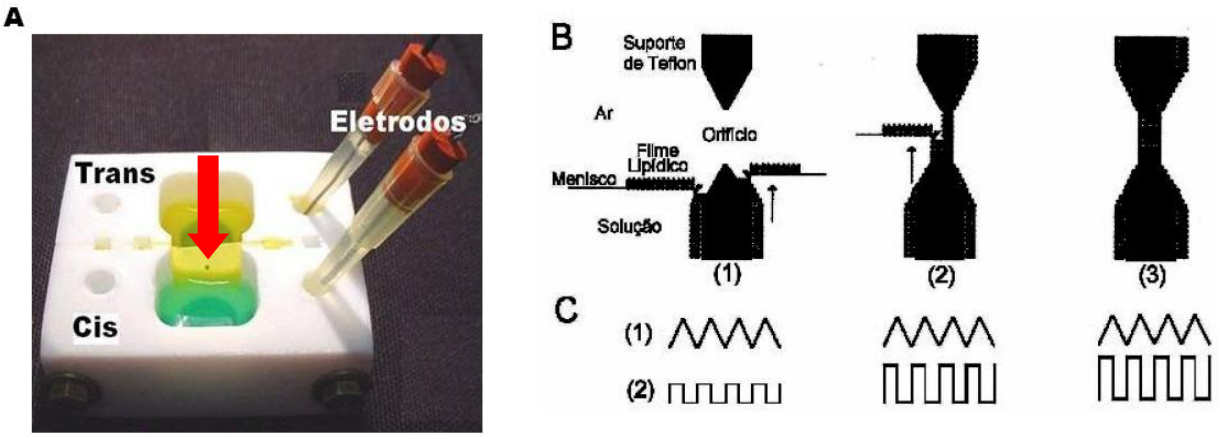


Figura 4. Montagem experimental para a gravação de nanoporo unitário em bicamada lipídica plana. (A) Câmara de Teflon® formando dois compartimentos (CIS e TRANS) onde são adicionadas as soluções iônicas e é formado o filme lipídico. A seta vermelha mostra o orifício no filme também de Teflon® onde é formada a bicamada lipídica. Os eletrodos de Ag/AgCl (prata/cloreto de prata) estão ligados ao aparato eletrônico que decodifica o sinal de corrente. (B) Formação da bicamada lipídica pela técnica de Montal e Mueller (MONTAL & MUELLER, 1972). (C) Corrente capacitiva visualizada no osciloscópio quanto à formação da bicamada lipídica. (1) é a onda triangular aplicada à membrana e (2) é a onda quadrada de resposta. Notar que quadrada aumenta de amplitude à medida que a membrana vai sendo construída (aumento da resposta capacitiva).



2.3. O NANOPORO PROTEICO COMO SEQUENCIADOR DE ÁCIDO DESOXIRRIBONUCLÉICO (DNA)

O início do desenvolvimento dos sequenciadores de DNA, sem dúvida se deu com Frederick Sanger, ganhador do prêmio Nobel de Química em 1980 , que desenvolveu métodos para a utilização in vitro da síntese de DNA na presença de dideoxiribonucleotídeos (ddN) para gerar fragmentos parciais de DNA que diferem por simples bases nucleotídicas, permitindo a determinação da sequência do polímero inteiro (SANGER et al., 1977). O método de sequenciamento de Sanger foi submetido a uma notável evolução ao longo de toda a "era genômica", principalmente pela enorme importância da decodificação das sequências de nucleotídeos. Foram implementadas modificações nas técnicas de sequenciamento a fim de se obter um tipo de sequenciador de alto rendimento e precisão, diminuindo o tempo e erros gerados durante os sequenciamentos.

No início dos anos 90, deu-se início à verdadeira "era genômica", com relatos iniciais de colaborações para sequenciar o genoma de *E. coli* (YURA et al., 1992) e *C. elegans* (SULSTON et al., 1992). Daí iniciou-se o planejamento para um o lançamento de um grande empreendimento de sequenciamento, o genoma humano (COLLINS & GALAS, 1993). Estes grandes projetos de sequenciamento começaram utilizando o sequenciamento de Sanger, posteriormente utilizando modificações na técnica. Nesta época houve um grande aumento na eficiência dos sequenciamentos com novas tecnologias para leitura de sequencias, modificações nas matrizes para eletroforese, manipulação robótica de líquidos, melhora química de corantes fluorescentes e nos detectores de sinais. Estas mudanças foram desenvolvidas para atender a demanda mundial de sequenciamento de DNA, tornando-a mais efetiva.

A técnica bioquímica de Sanger é considerada "o primórdio" dos sequenciadores de DNA, e vem sendo substituída por novas tecnologias que prometem informação da sequência de maneira mais rápida e barata. A partir da necessidade de novas tecnologias para a otimização do sequenciamento, foram criadas novas técnicas que são chamadas de sequenciamento de "segunda geração". Novas alternativas de sequenciamento de DNA podem ser agrupadas em categorias (SHENDURE & JI, 2008):

I. Métodos microeletroforéticos: Houve progresso significativo para desenvolver métodos que permitam que os sequenciamentos eletroforéticos convencionais sejam realizados em um micro dispositivo. As principais vantagens dessa abordagem incluem o processamento mais rápido e uma redução substancial no consumo de reagentes. Para criar

este dispositivo ideal seria necessário integrar todos os aspectos do processamento da amostra, por exemplo com o transporte microfluídico dos reagentes entre as etapas; a amplificação por PCR clonal na escala de nanolitros a partir de uma única célula ou uma molécula modelo única; purificação; ciclo de reação de sequenciamento; isolamento e concentração de fragmentos de extensão; e injeção em um microcanal de separação eletroforética (PAEGEL et al., 2003; HONG & QUAKE, 2003);

II. Sequenciamento por hibridização: O princípio básico deste método de sequenciamento consiste em fragmentos do ácido nucléico marcados hibridizados diferencialmente em uma matriz de sondas de oligonucleotídeos, que podem ser usados para identificar com precisão as posições variantes dos nucleotídeos. Normalmente, os oligonucleotídeos presos à matriz são concebidos como uma representação da sequencia de referência correspondente ao genoma de interesse. Esta técnica encontra como competidor direto a técnica de microarranjo ("*microarray*"), que possui os mesmos princípios da hibridização, porém são usados mais frequentemente para o ressenquenciamento do genoma de interesse em menor escala. As duas técnicas começaram a competir pelas mesmas aplicações, como por exemplo, ressequenciamento, análise da expressão, análise de variações estruturais, ligação de DNA a proteínas. Em termos de sequenciamento o microarranjo possui limitações: (i) sequencias que são repetitivas ou sujeitas a hibridização cruzada não podem ser facilmente encontradas, (ii) não se sabe como sequenciamento *de novo* pode ser alcançado com estratégias baseadas em hibridização, e (iii) sem a análise cuidadosa dos dados, os falsos positivos representam um problema importante, e não está claro como obter o equivalente à cobertura redundante que é possível com o sequenciamento convencional de matriz-cíclica. O sequenciamento por hibridação teve seu maior impacto, provavelmente, no contexto dos estudos de associação do genoma, que dependem de genotipagem por hibridização de um grande conjunto definido de coordenadas genômicas adjacentes (PIHLAK et al., 2008);

III. Sequenciamento por arranjo cíclico: O conceito de sequenciamento por arranjo cíclico pode ser resumido como o sequenciamento de um arranjo denso de DNA, caracterizado por ciclos repetitivos de manipulação enzimática e coleta de dados baseados em imagens. Embora essas plataformas sejam bastante diversificadas do sequenciamento bioquímico de Sanger, tanto na forma como no arranjo que é gerado, seus fluxos de trabalho são conceitualmente semelhantes. Neste método, adaptadores comuns são ligados ao DNA genômico fragmentado, o que é então submetido a um dos vários protocolos que resulta em um arranjo de milhões de colônias de PCR espacialmente imobilizadas em uma matriz. Cada colônia consiste em muitas cópias de um único fragmento alvo. Como todas as colônias estão amarradas a uma matriz planar, um volume único de reagente na escala de microlitro pode ser

aplicado para manipular todos os recursos da matriz. Da mesma forma, a detecção de imagens baseada em etiquetas fluorescentes incorporadas a cada extensão pode ser utilizada para adquirir dados de sequenciamento. Sucessivas iterações enzimáticas e de imagem são usadas para construir uma sequência contínua de leitura para cada característica do arranjo (ROTHBERG & LEAMON, 2008);

IV. Sequenciamento em tempo real de fita simples: Vários grupos acadêmicos e empresas estão trabalhando em tecnologias de sequenciamento de DNA ultra-rápidos, que são substancialmente diferentes da atual safra de plataformas disponíveis da "segunda geração" (DEAMER & AKESON, 2000). Uma abordagem envolve o acompanhamento em tempo real da atividade da enzima DNA polimerase. A incorporação de nucleotídeos pode ser detectada através de FRET (fluorescência de transferência de energia de ressonância) que ocorre devido a interações entre uma polimerase ligada a um fluoróforo e nucleotídeos marcados com marcação fluorescente, de tal forma que a incorporação de nucleotídeos pode ser monitorada (COCKROFT et al., 2008).

Outra abordagem é o sequenciamento baseado em nanoporo, no qual os ácidos nucleicos são conduzidos através de um único nanoporo, formado pela incorporação de uma proteína (tal como a α-hemolisina) em uma matriz lipídica, ou um poro sintético contruído em suporte não biológico. Flutuações da condutância de DNA através do poro, ou, eventualmente, a detecção de interações de bases individuais com o poro, são usados para detectar a sequência de nucleotídeos. Apesar dos progressos alcançados na realização do método, os principais desafios técnicos permanecem no caminho para uma plataforma de sequenciamento verdadeiramente prático (MELLER et al., 2000).

Neste trabalho abordamos o aperfeiçoamento do nanoporo para o sequenciamento de segunda geração em tempo real de fita simples, chamado também de sequenciamento "ultra-rápido", ou seja, maior rapidez de análise e a princípio ausência de marcação e amplificação do material a ser sequenciado (DEAMER, 2010; MAGLIA et al., 2010). Este sequenciamento é realizado usando uma tecnologia baseada em nanoporo, que pode ser de origem proteica ou sintética, chamados nanoporos de estado sólido (LU & YU, 2012).

A primeira estrutura de nanoporo proteico usado para o sequenciamento de DNA foi a α-hemolisina e continua até hoje sendo a mais indicada para esta tarefa. O que a faz ter tanta aplicabilidade é sua fácil obtenção e modificação, e o conhecimento de sua cinética e interação com bicamadas lipídicas de diferentes tipos (KASIANOWICZ et al., 1996; VALEVA et al., 1997). Menos de dez anos depois do primeiro ensaio com a α-hemolisina, foram realizados experimentos usando uma proteína também proveniente de bactérias, a

porina A da *Mycobacterium smegmatis* (MspA) (FALLER et al., 2004). Esta proteína, semelhante à α-hemolisina (Figura 5), forma poros em membrana e possui a capacidade de detecção do DNA, sendo de grande importância na detecção de sequencias curtas (MANRAO et al., 2012). Além destas proteínas, a proteína G (OmpG), quando modificada por mutação, possui potencial para ser usada como nanoporo para detecção do nucleotídeo adenosina difosfato (ADP) (CHEN et al., 2008).

Além de nanoporos biológicos, são utilizados também os nanoporos de estado sólido os quais são baseados em materiais sintéticos que são de grande aplicabilidade na pesquisa. Os mais utilizados são os nanoporos de grafeno (**Figura 5**) (GARAJ et al., 2010) e os de materiais cerâmicos (ANANDAN & OKAZAKI, 2005)

Na metodologia de sequenciamento por nanoporo, uma única fita simples de DNA é translocada eletroforeticamente através do lúmen de um único nanoporo da α-hemolisina banhada por solução aquosa iônica. As bases são discriminadas por induzirem padrões característicos na série temporal da corrente iônica durante a passagem. Essa tecnologia do nanoporo possibilitará medidas da estrutura dos ácidos nucleicos, com uma precisão em escala nanométrica. A técnica promete ser rápida, sem reagentes ou marcadores químicos e de menor custo, se comparada aos métodos convencionais. Vários grupos de pesquisa vêm em tentativas de obter êxito nesta área, porém ainda há necessidade de vários aperfeiçoamentos na técnica (WANUNU & MELLER, 2007; WANUNU et al., 2008; DEAMER, 2010; DERRINGTON et al., 2010; MAGLIA et al., 2010; STODDART ET AL., 2010b; ZHAO, 2010).

Dentre os vários problemas apresentados na técnica, há um problema fundamental: O insuficiente nível de reconhecimento das bases durante a passagem da molécula do DNA via nanoporo. Isto se deve à altíssima velocidade de translocação da molécula de DNA através do poro (≤10μs por base). Este valor está no limite atingível experimentalmente e insuficiente para detectar individualmente, um nucleotídeo. A velocidade desejável para o sequenciamento com o nanoporo é 1ms por base, quando será possível deduzir a sequência de nucleotídeos com precisão, analisando a corrente residual (**Figura 6**). O problema pode ser abordado de várias maneiras, como por exemplo, engenharia molecular e a melhoria do design experimental (MUTHUKUMAR, 2007).

Figura 5. Comparação das dimensões e estruturas dos diferentes nanoporos. A primeira imagem mostra a estrutura do nanoporo da α-hemolisina incorporado a uma membrana lipídica. A segunda, a estrutura de uma nanoporo de MspA (porina A da *Mycobacterium smegmatis*) incorporado a uma membrana lipídica. A terceira é a estrutura de um nanoporo de grafeno. Adaptado de SCHNEIDER & DEKKER, 2012.

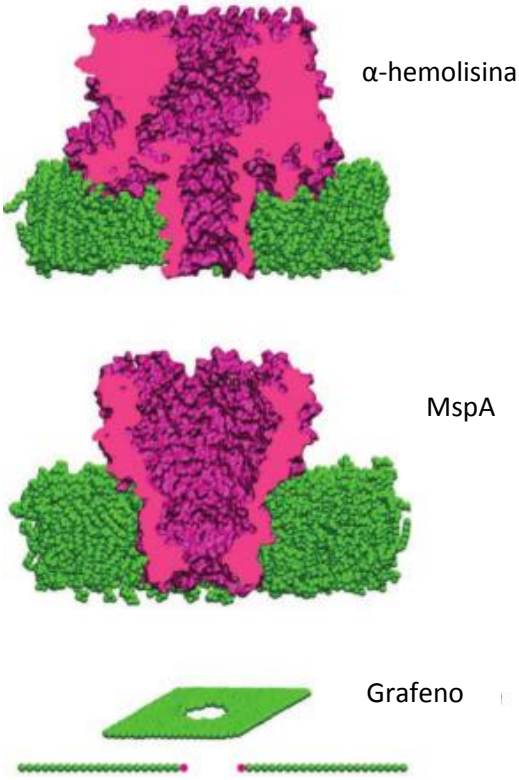
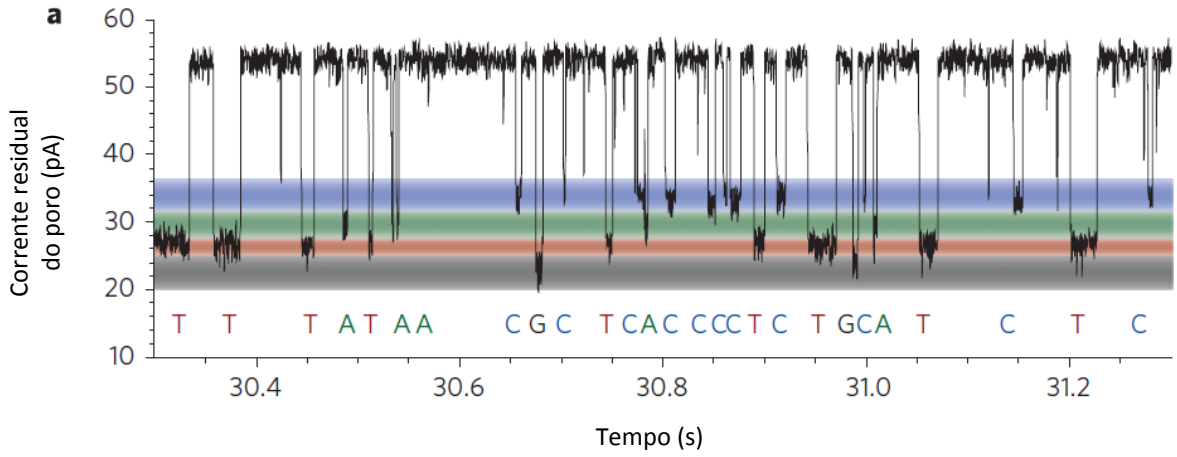


Figura 6. Representação do sequenciamento com o nanoporo da α-hemolisina mostrando as diferenças que existem nos eventos de bloqueio de corrente para cada base do DNA (T, A, G e C). Adaptado de CLARKE et al., 2009.



Uma das estratégias para diminuição da velocidade de translocação dos ácidos nucleicos é o emprego de substâncias que alteram o grau de estruturação da água e que, portanto, potencialmente podem modificar alguns parâmetros, tais como, viscosidade do meio e energia de interação do DNA com o nanoporo (RODRIGUES et al, 2011; KOWALCZYK et al., 2012). Outra manobra a ser usada, são as alterações na estrutura proteica do nanoporo, realizando a diminuição do diâmetro do poro ou adição de cargas, onde se pretende "frear" a passagem do ácido nucléico, para obter melhor resolução (CLARKE et al., 2009; RINCON-RESTREPO et al, 2011;WEN-WU et al, 2011).

2.4. NANOPOROS ADAPTADOS

Apesar de todos os avanços com os nanoporos como detectores de diversos tipos de analitos e sequenciadores de ácidos nucleicos, temos a limitação da translocação através do nanoporo ser muito rápida para moléculas que possuem carga elétrica elevada. Vários trabalhos passaram a ser desenvolvidos realizando alterações na técnica, a fim de encontrar melhores condições experimentais para o aperfeiçoamento do processo de detecção.

Para melhorar a detecção, não apenas dos ácidos nucleicos e nucleotídeos, as mudanças realizadas nos parâmetros experimentais vão de alterações na composição da solução banhante: troca de íons (RODRIGUES et al., 2011); concentração dos íons (RODRIGUES et al., 2008); alteração do pH (JEON & MUTHURKUMAR, 2013). Estas alterações na solução banhante podem ser aliadas às mudanças na composição das membranas (POULOS et al., 2009), mutações e funcionalizações do elemento sensor (ASTIER et al., 2006; GUAN et al., 2005; KANG et al., 2006).

As modificações na estrutura do elemento sensor podem ser realizadas geneticamente ou quimicamente (KRASILNIKOV et al., 2000; MERZLYAK et al., 2005; CLARKE et al., 2009; BANERJEE et al., 2010). A α-hemolisina é uma proteína de fácil adaptação e funcionalização, por ter a sua estrutura cristalográfica bem elucidada (SONG et al., 1996) e a cinética de inserção em membranas bem descrita (VALEVA et al., 1997; RODRIGUES, 2006).

Os nanoporos protéicos mutantes podem ser produzidos utilizando a técnica de mutagênese sítio-dirigida. Na mutagênese sítio-dirigida um aminoácido ou um grupo de aminoácidos que compõe a estrutura protéica do nanoporo pode ser substituído por outros aminoácidos gerando uma variedade de cadeias laterais de diferentes tamanhos, formas,

polaridade e reatividade (STODDART et al., 2010a; STODDART et al., 2010b). Também podem ser realizadas mutagênese introduzindo aminoácidos não naturais, com a finalidade de sintetizar cadeias laterais que não ocorrem na natureza.

Os nanoporos também podem ser modificados quimicamente. Nesta modificação um aminoácido da cadeia lateral, geralmente cisteína, é seletivamente modificado com um reagente químico permitindo a incorporação de uma diversidade de grupos funcionais que podem ser incorporadas dentro do nanoporo. E, esta técnica, chamada de *cisteine scanning*, é realizada com a utilização de reagentes a base de metanoetil sulfonato (MTS) (MERZLYAK et al., 2005). As α-hemolisinas projetadas ("engenheiradas") têm sido estudadas com a finalidade de tornar mais específica a detecção do analito pelo biossensor. O uso desses nanoporos protéicos "engenheirados" abrange potenciais aplicações desde a detecção ultrassensível de moléculas unitárias até o sequenciamento de DNA (SHIM & GU, 2007; MOVILEANU, 2009; STODDART et al., 2010a). Além disso, há estudos para modular a seletividade e a sensibilidade do poro, com o uso de adaptadores moleculares introduzidos no interior do nanoporo formado pela α-hemolisina (**Figura 7**), tornando ainda mais estreita a sua constrição, e desse modo, favorecendo uma seletividade específica com intuito de facilitar a identificação e a quantificação do analito (BANERJEE et al., 2010). Esta adaptação pode ser realizada tanto de forma não-covalente (ASTIER et al., 2006), como covalente (WU et al., 2007; CLARKE et al., 2009; MARTIN et al., 2009).

Considerando as dificuldades apresentadas para a melhoria do sequenciamento com nanoporo, o processo de transporte de ácidos nucleicos através de um nanoporo tem atraído a atenção de muitos químicos teóricos (AKSIMENTIEV, 2010), que trouxeram para o campo diversas experiências das áreas da física. Por conseguinte, o espectro de métodos usados para modelar este processo é amplo, dependendo do tipo de sistema e nanoporo usado. Na próxima seção, um dos métodos mais populares da química computacional para a análise de transporte em sistemas de nanoporos, é brevemente descrito.

Figura 7. Exemplo de nanoporo mutante (pontos em vermelho indicam os locais de mutação) para favorecer a colocação de um adaptador molecular, neste caso uma β-ciclodextrina modificada foi acoplada covalentemente no interior do poro protéico (molécula em verde). Adaptado de CLARKE et al., 2009.

2.5. QUÍMICA COMPUTACIONAL DE BIOMOLÉCULAS

A computação baseada em modelos moleculares é uma importante ferramenta nas áreas biológicas em geral, incluindo a bioquímica e a biofísica. Como as medidas de um número limitado de propriedades de um sistema biomolecular são acessíveis através da parte experimental, as simulações computacionais podem complementar, trazendo não apenas médias, mas também distribuições e séries temporais, definindo quantidades. Por exemplo, podemos obter resultados de distribuições conformacionais e interações entre partes de um sistema biológico (VAN GUNSTEREN et al., 2006).

Simulações de dinâmica molecular capturam o comportamento de macromoléculas biológicas detalhadamente a nível atômico. Através destas ferramentas podemos e calcular vários parâmetros envolvidos nos principais processos bioquímicos, como o enovelamento de proteínas, ligação de drogas a receptores, transporte através de membranas e as mudanças conformacionais importantes para a função proteica. Tais simulações podem servir como um "microscópio computacional", revelando mecanismos biomoleculares em escalas espaciais e temporais que são difíceis de observar experimentalmente (DROR et al., 2012).

No nosso sistema, através das técnicas de gravação de canal unitário, podemos obter diversas informações quanto à interação do analito com o nanoporo e o sistema. Ainda assim, há informações a níveis microscópicos que são não podem ser precisamente inferidas com estas técnicas. Experimentos que investigam a translocação de ácidos nucleicos sob a influência de um potencial transmembrana sugerem que o processo demora tipicamente de centenas de microssegundos a dezenas de milissegundos (AKESON et al., 1999). Para

entender os processos inerentes à cinética e termodinâmica da translocação de uma fita de ácido nucleico através do nanoporo de α-hemolisina e melhorar a técnica de gravação de canal unitário, podemos usar simulações de dinâmica molecular do processo de translocação obtendo informações cinéticas e estruturais difíceis de serem obtidas apenas através dos experimentos.

As simulações de sistemas fora do equilíbrio permitem que se reduza a escala de tempo na qual os fenômenos físicos acontecem, podendo ser investigados com resolução atomística. Vários tipos de simulações fora do equilíbrio podem ser concebidos para efetuar a translocação a velocidades mais elevadas, permitindo que o processo de translocação completo seja estudado. A dinâmica molecular direcionada (*"steered molecular dynamics"*) (PHILLIPS et al., 2005) é um tipo de simulação fora do equilíbrio em que se aplica uma força constante a um átomo ou grupo de átomos, ou conecta um átomo ou centro de massa de um grupo de átomos através de um potencial harmônico de restrição da posição, regulado por uma força, que é movido a uma velocidade constante. A dinâmica molecular direcionada de velocidade constante (SMD-cv) tem a vantagem de ter um prazo bem definido de simulação por uma distância de translocação dada; SMD-cv é usada para induzir a alta velocidade de translocação, como experimentalmente (MARTIN et al., 2009), e assim podemos analisar um sistema o mais próximo do real.

O cálculo da energia livre é um dos mais importantes aspectos no campo da biologia computacional, pois serve como uma ponte crítica entre os dados teóricos e os experimentais. Com a energia livre proveniente dos experimentos podemos avaliar a confiança do modelo teórico e aperfeiçoar a precisão do modelo. Por outro lado, a energia livre computada no modelo teórico se torna uma ferramenta complementar às informações obtidas experimentalmente (BOND et al., 2011). O perfil de energia livre, pode ser considerado como uma referência em muitos processos reais de transição ou reação, tais como o dobramento de proteínas (FINKELSTEIN & GALZITSKAYA et al., 2004); o transporte de íons (PICCININI et al., 2007); afinidade relativa de ligação proteína-ligante (SUEVER et al., 2008); e afinidade relativa de medicamentos em potencial (BÖHM, 1998).

Nós exploramos as simulações de dinâmica molecular para ter uma visão microscópica da translocação do ácido nucleico através do poro proteico a partir do cálculo de seus perfis de energia livre. É desejável que se obtenha o perfil energia livre do processo de translocação devido ao seu papel central na determinação das propriedades termodinâmicas do sistema, e que também pode ser usada para determinar as propriedades cinéticas. A energia

livre é uma propriedade de equilíbrio; a fim de calcular a energia livre a partir de um processo fora do equilíbrio, tal como SMD-cv, pode-se usar a igualdade de Jarzynski (JE) (JARZYNSKI, 1997). Ao obter e analisar os perfis de energia livre do processo de translocação são reveladas informações sobre as barreiras de energia que uma molécula de nucleotídeo tem experimentalmente e, consequentemente, o que geram essas barreiras.

A igualdade de Jarzynski (JE) é uma técnica que foi descrita inicialmente por Jarzynski em 1997, é usada para extrair as energias livres a partir de processos fora do equilíbrio. A JE iguala a mudança da energia livre, que ocorre no equilíbrio, à média do conjunto dos trabalhos realizados em repetições do mesmo processo, como descrito na equação 1:

$$e^{-\beta \Delta F} = \langle e^{-\beta W} \rangle \quad (1)$$

Onde β é o inverso da temperatura multiplicado pela constante de Boltzmann ($1/k_B T$); os parênteses angulares representam a média termodinâmica; ΔF é a variação de energia livre; e W é o trabalho em cada realização do processo. Sendo assim a igualdade de Jarzynski representa a igualdade entre a energia livre em equilíbrio e a média do trabalho realizado em um processo fora do equilíbrio.

Neste trabalho empregamos química computacional por meio das técnicas de Dinâmica Molecular, como uma ferramenta para avaliação da modificação estrutural do nanoporo de α-hemolisina, análise do perfil de energia livre resultantes de sua interação com ácidos nucléicos, visando à predição de qual nanoporo adaptado seria teoricamente o mais apto para funcionar como sequenciador de DNA.

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4. OBJETIVOS

4.1. OBJETIVO GERAL

Investigar, *in silico*, a influência das modificações na estrutura proteica do nanoporo de alfa-hemolisina na interação com ácidos nucleicos.

4.2. OBJETIVOS ESPECÍFICOS

- Realizar triagem por meio de software de modelagem computacional para analisar como as alterações na estrutura proteica (mutações e funcionalização) podem influenciar no diâmetro do poro.
- Determinar os parâmetros ideais na realização de dinâmica molecular para observar o potencial de força média que ocorre nas interações entre o ácido nucleico e o nanoporo de α-hemolisina, comparando as formas mutantes com a forma nativa (WT).
- Prever alterações no diâmetro e no perfil eletrostático do nanoporo mutado da α-hemolisina em preparação para o uso de adaptadores moleculares.

CAPÍTULO 1

STEERED MOLECULAR DYNAMICS SIMULATIONS OF DNA TRANSLOCATION THROUGH THE α-HEMOLYSIN NANOPORE

Annielle M. B. da Silva, Cláudio G. Rodrigues, Adriana Fontes and Gustavo Seabra

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Steered Molecular Dynamics Simulations of DNA translocation through the α-hemolysin nanopore

Annielle M. B. da Silva, Cláudio G. Rodrigues, Adriana Fontes and Gustavo Seabra

Abstract

Staphylococcus aureus produces α-hemolysin protein that, when incorporated into a planar lipid bilayer, forms a nanopore that has been used for detection of several molecules and is considered a promising biosensor. In the experimental devices, the "reading" of DNA sequences is possible by electrophoretically driving the translocation of single-stranded DNA through the nanopore. However, the extremely high speed of nucleotides translocation is a problem for the secure identification of nucleotides. In this work, nonequilibrium molecular dynamics simulations are performed to study the process of DNA translocation trough the wild α-hemolysin nanoporo, and two selected mutants. Understanding the translocation will direct experimental studies for improving detection in future nucleic acid sequencers.

Introduction

Detection and/or monitoring of specific molecules in solution are of fundamental importance in basic science and in technological applications (1). Conventional sensors are usually quite slow and sensitive only to relatively large quantities of molecules of interest. Nanopore stochastic sensing is currently an active research area, characterized by highly-sensitive, rapid and multifunctional detection capabilities (2). The sensing principle of such sensors is based on transient interruptions in the ion-current of an electrolyte, induced by the entry, transport, and exit of a particular analyte from the pore. The interaction events are randomly distributed on the time scale that permits to designate such sensors as stochastic. The basis of nanopore sensors is the same of conventional Coulter counter invented in 1953 (3). The size of the sensing element is the main difference. There is no fundamental limit to minimum detectable particle size, but for optimal detection the analyte should be on the order of the pore size. There are several studies aimed to develop a molecular Coulter counter using nanopores formed in different materials: inorganic, polymer films (4–8) and, of course, formed protein nanopores in lipid bilayers (1, 9–11). The nanopore formed by α-hemolysin of *Staphylococcus aureus* (αHL) is most used so far for this aim (12–27)

Despite the proof-of-concept for the use of single nanopores in sensor applications, single molecule detection level and DNA sequencers, the improving the detection capability of the nanopore is the barest necessity. In the approach of sequencing by nanopore sensing technology means that a single stranded molecule of DNA or RNA is electrophoretically driven through a single nanopore, and each base is read as it passes at a point of recognition within the nanopore. The current that is generated by the passage of ions (e.g., K^+ and Cl^-) through the nanopore during DNA translocation provides the reading needed to distinguish each base. The nanopore formed by the bacteria *Staphylococcus aureus*'s α-hemolysin protein, when incorporated into a planar lipid bilayer, is regarded as one of the most promising bioestructures to the production of molecular sequencers, once it has been fully elucidated its molecular crystalline structure (28) since it has geometric dimensions compatible with DNA, and ease of adaptation by genetic engineering techniques and/or chemical modification.. The major challenge to α-hemolysin nanopores use as nucleic acid sequencer is the high translocation speed of the DNA strand through the nanopore, which

avoids the detection of the different nitrogen bases to occur efficiently and reliably. In this direction, it is desirable to find ways to reduce the rate of DNA strand translocation and thereby increase the residence time of the protein within the nanopore. Strategies for overcoming this operating challenge are changing the ionic solution composition or concentration (26, 29, 30); or changing the protein structure, by site-directed mutations or chemical functionalization (31–33). The aim could be achieved in several ways, e.g., via molecular engineering of the nanopore and improvement of experimental design (10, 34–39)

A pioneering work by Akabas and colleagues (40) demonstrated that cysteine scanning mutagenesis could be used to map the topology of ion channels (13). Later, Merzlyak and colleagues demonstrated the use of cysteine scanning to carry mutations in specific regions of the α-hemolysin nanopore, and subsequently introduced chemical modifications in these novel cysteine side chains with water-soluble sulfhydryl-specific reagents, suggesting the net charge of the channel wall to be responsible for cation-anion selectivity of the α-hemolysin nanopore (10).

We applied nonequilibrium molecular dynamics simulations to get a microscopic view and obtain the free energy profiles of the translocation of a single-stranded DNA through the α-hemolysin pore. Some of the mutants used in this last work(31)were submitted to constant velocity steered molecular dynamics (cv-SMD) simulations, and the Jarzynski relation was used to obtain the free energy of the process.

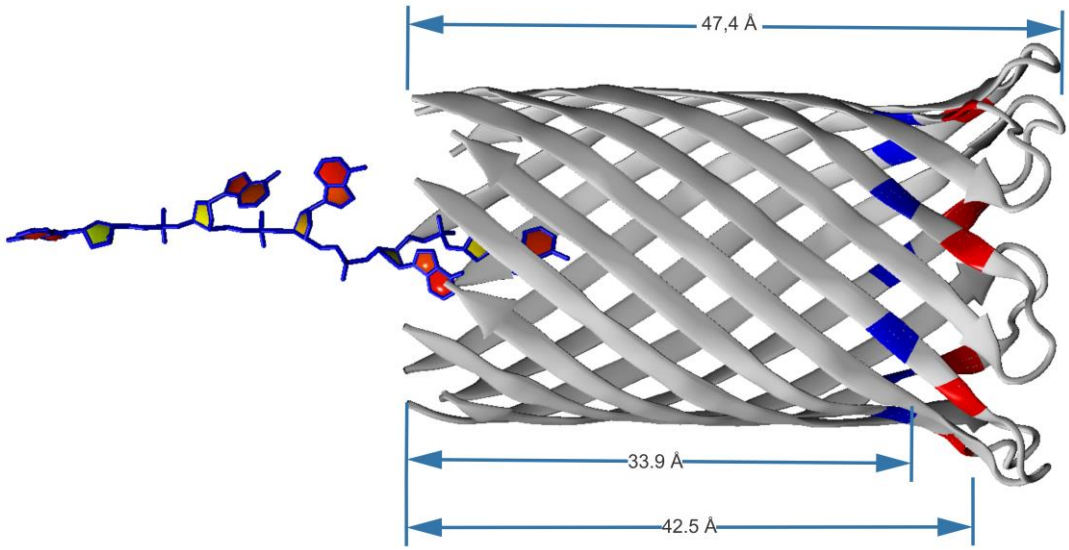

Figure 1. Figure representing the starting configuration of 5’ led 5 poly(dA) nucleotide translocation simulation. Colored residues in the pore point to the mutation positions: blue-colored residues in the pore indicate position 135, while the red-colored residues indicate position 133 (respectively Leucine and Glycine, in wild-type α-hemolysin).

Materials and Methods

Mutants Selection

The α-hemolysin crystallographic structure coordinates were taken from Protein Data Bank (PDB) entry 7AHL(28). In the work by Merzlyak et al mentioned above,(31) site-directed mutagenesis was used to replace the native amino acid for a cysteine at 24 different positions, and subsequent addition of sulfidryl-specific reagents that bind to cysteine's free –SH group. Among those positions, we chose six as possible candidates for the computational study. The criteria for the selection included that the amino acid side-chain must be pointed towards the inside of the channel. Thus, odd-numbered positions 129, 133 135, 139, 141 and 143 were chosen for mutation, which were applied in the 7 α-hemolysin subunits.

PyMOL Molecular Graphics System (41) software was used to apply the mutations, using the available *mutagenesis* tool. The diameter of the channel was measured using the *measurement* tool. For each structure, 7 distances were measured, between the innermost atoms of the amino acids side-chains in diametrically opposing positions.

Model Construction and Simulation Details

Starting from the PDB structure, a minimal model of the system was built by keeping only the residues that are inserted in the membrane, i.e., the *stem* (residues 111–147 in each of the seven chains that make the pore) (Figure 1). Atoms missing in the original PDB structure (such as hydrogen atoms) were included during model building by the LEaP program, part of the AMBER 12 Molecular Dynamics Suite.(42) The DNA oligomer of 5 adenine bases was also built using LEaP, and manually inserted into the nanopore using UCSF Chimera.(Figure 1) The selected mutants were constructed by mutating the corresponding residues on this last structure.

All simulations were performed using the molecular dynamics simulation program *sander*, part of the AMBER 12 package. Before molecular dynamics all structures were subjected to 10.000 steps of energy minimization. To account for solvation effects, we use the generalized Born model-2, developed by A. Onufriev, D. Bashford and D.A. Case.(43) We restrain the α-carbon atoms (C_α) of the protein stem with a low force constant of 3.0 kcal mol^{-1} $Å^{-2}$, to simulate the presence of a lipid membrane externally to nanopore and to prevent the entire pore from being dragged during the cv-SMD simulations.

As shown by several groups, a difference is observed in the translocation times of nucleic acid polymers depending on whether they are threaded by 3' or 5' end of the nucleotide chain. (44, 45). We have chosen to run the translocation simulations by steering the C3' carbon atom at the 5' tail residue of the DNA through the barrel of α-hemolysin. This atom was aligned at a 9 Å distance to the center of mass of all the C_α of protein residues 111, which form a ring on the top of the stem. (Figure1)

The Visual Molecular Dynamics software [VMD, (46)] was used to count the number of hydrogen bonds between the DNA molecule and the protein during DNA translocation. H-bonds were counted if donor-acceptor distance was below 3.0 Å, and the angle below 45°. This procedure was applied to all simulations, and the number of H-bonds averaged over all repetitions of the same system.

Jarzynski's Relation

The calculation of free energy is one of the most important aspects in the field of computational biology, serving as a critical bridge between the theoretical and experimental data. With the free energy from the experiments we evaluate the confidence of the theoretical model and improve model accuracy. Moreover, the free energy computed in the theoretical model becomes a complementary tool to experimental information (47). The free energy profile can be regarded as a reference in many structural or reactional processes such as protein folding (48); ion transport (49); protein-ligand relative affinity binding (50)and relative affinity of potential drugs(51).

Understanding the free energy profile of the DNA translocation process is important to determine thermodynamic and kinetic properties of the system. The free energy is an equilibrium property and, in order to calculate it from a non-equilibrium process as cv-SMD, the Jarzynski's relation (JR) can be used (18). The JR equals the change in free energy that occurs at equilibrium, to the ensemble average of the work performed on a large number of realizations of the same process as described in equation 1:

$$e^{-\beta \Delta F} = \langle e^{-\beta W} \rangle \quad (1)$$

Here, β is the inverse of temperature multiplied by the Boltzmann's constant ($1/k_B T$), the angled brackets represent the ensemble average; ΔF is the change in free energy, and W is the work for each process. Thus the Jarzynski's relation is the equality between the free energy in the equilibrium and the average of the work done in the multiple repetitions of a non-equilibrium process.

Results and Discussion

Mutant Selection

The average of the distances between the innermost atoms of the amino acids side-chains in diametrically opposing positions for the six mutants is shown in Table 1**Erro! Fonte de referência não encontrada.**. From those, we decided to simulate systems with the mutations on aminoacids 133 and 135, respectively the shortest and longest predicted diameters, leading to mutants G133C and L135C. The first one resulted in a 4 Å reduction in diameter, expected to increase the DNA residency time inside the pore. In the second, a leucine was replaced by cysteine, resulting in diameter increase. Previous work had already determined the importance of these positions in DNA interaction. (52)

Position – Aminoacid	WT	Mutant
129 – T	24.46 ±0.43	24.94±0.36
133 – G	24.47±0.34	20.71±0.38
135 – L	16.67±0.63	19.07±0.21
139 – N	20.2±0.35	20.46±0.51
141 – S	20±0.59	20.34±0.52
143 – G	20.06±0.30	19.61±0.23

Table 1. Diameter measurement. The WT column represents the diameter measurement of the wild type α-hemolysin. The Mutant column represents the diameter measurement of mutants by cysteine addiction

Optimization of SMD parameters

There are many parameters that need to be carefully selected for effective SMD simulations. Here, the spring constant and pulling speed were selected before simulations, because these were especially sensitive to the size and conformational behavior of the pulled molecule, requiring explicit attention for the translocation of DNA.

If the system is too far from equilibrium, the translocations of large deformable molecule in a SMD simulation can result in undesirable conformational changes. This is due to translocation forces being applied to only a small part of molecule. If the molecule moves too fast, there is insufficient time for relaxation forces to return the structure to its equilibrium state.(53)

Figure 2 shows the effect of the SMD pulling speed on the separation of two DNA bases, as measured by the distance between the center of masses of the atoms C3' and H3' from the first and second DNA residues. It is visible that, at large pulling speeds (5.0 and 2.0 Å ps^{-1}), the distance increases continually, indicating the stretching of the DNA. At pulling speeds of 1.0 Å ps^{-1} and below the distance between the bases stabilizes. We have chosen to use a pulling speed of 1.0 Å ps^{-1}, for offering a good compromise between speed and simulation time.

Figure 3 shows the effect of the force constant, as measured by the lag between the real position of the C3' atom and the center of the restraint, all measured at a pulling speed of 1.0 Å ps^{-1}. For small force constants, the discrepancy between the restraint and the steered atom positions is clear, while larger force constants are able to minimize the lag. Again, the value of 100 kcal mol^{-1} $Å^{-2}$ to be used was chosen based on a compromise between accuracy and computational time.

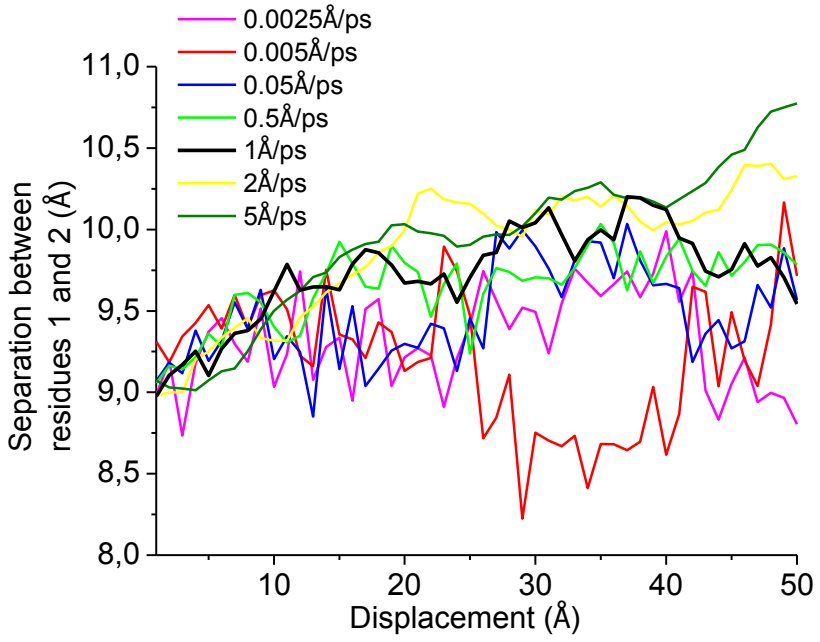

Figure 2. Effect of pulling speed in the separation of DNA residues.

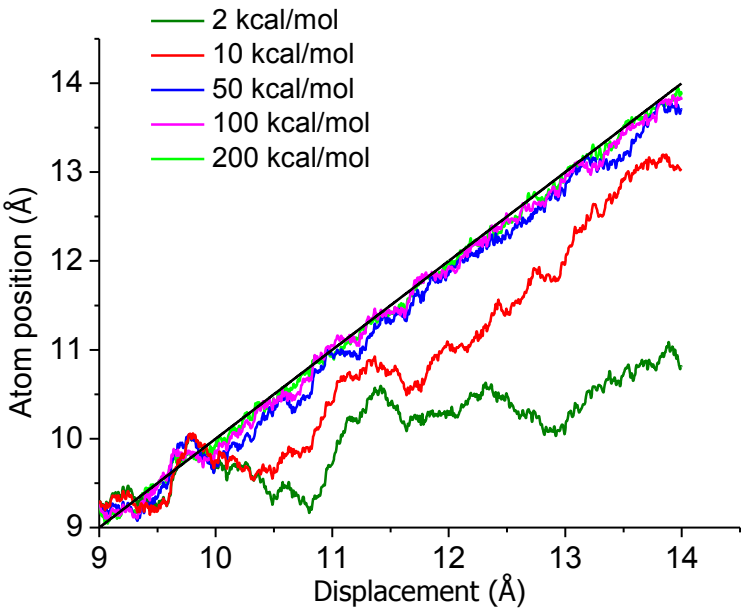

Figure 3. Effect of spring constant values on the lag between the SMD atom and the constraint position (displacement). The abscissa shows the required atom position (constraint), while the ordinate shows the real position. The solid black line indicates the ideal situation where the real atom position perfectly follows the constraint position.

Simulation of Oligonucleotide Translocation

We present the free energy profiles from the translocation of 5 poly(dA) nucleotide through the α-hemolysin nanopore in wild type and mutant form. The free energy profiles were obtained using the JR and can demonstrate interactions between molecules in a system. Figure 4 shows the potential of mean force (PMF) in DNA translocation though the α-hemolysin nanopore, that demonstrated the interactions between the DNA oligomer and amino acid side chains from the nanopore (54).

Comparing the PMFs, it is clear that both mutants already require a higher free energy for the translocation, with the L135C mutant showing the highest barrier, which happens precisely when the pulled atom reaches this position. In the wildtype α-hemolysin the 135 postion is comprised of seven leucines, all pointed towards the center forming a hydrophobic ring. Apparently, exchanging those amino acids by cysteines, smaller and more polar, increased the interaction between DNA and the ring, leading to a higher translocation barrier.

For the G133C mutant, on the other hand, there was an overall increase in the free energy profile, but a local reduction in the translocation barrier when the pulled atom reaches the point of mutation, which may be due to the restriction caused by replacing the glycines by cysteines.

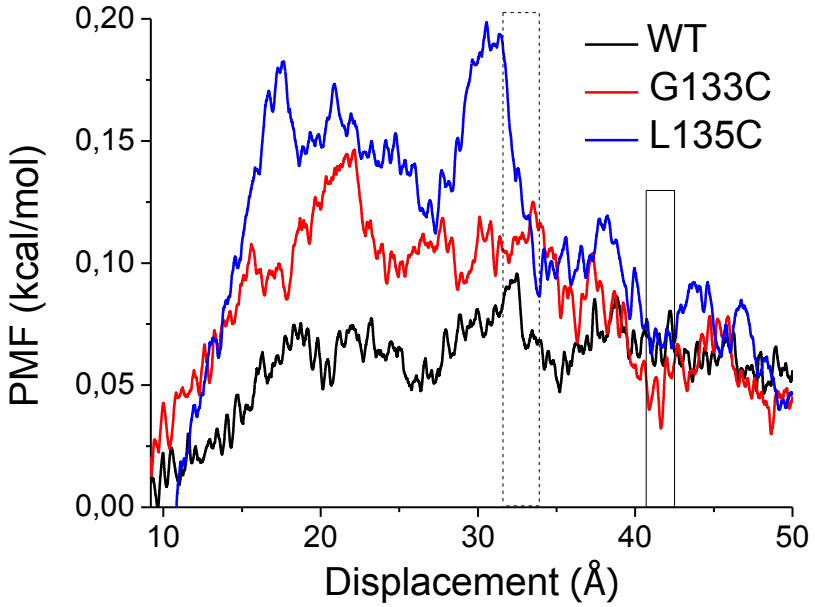

Figure 4. Potential of mean force for the translocation of DNA through the α-hemolysin nanopore, as a function of the displacement of the pulled atom. The dashed mark shows the 135 position and the solid mark shows the 133 position.

Figure 5 shows the average pore diameter as measured at the position of the mutations, throughout the simulation. It shows that the pore is stable and, although the individual measures of diameter vary during the simulation, the average diameter at the positions of interest are in agreement with the preliminary measurements (Table 1). These results indicate that the restrictions to hold the stem structure functioned as expected.

The average number of hydrogen bonds between the DNA and the protein structure during the translocation is shown in Figure 6. As expected, one can see that the increase in diameter for the L135C mutant resulted in a lower number of hydrogen bonds at the site of mutation. On the other hand, the G133C mutant shows a slight decrease in the number of H-bonds, which may also result from the decease in flexibility caused by the mutation.

Conclusion

We presented here a new model system for the simulation of DNA translocation through the α-hemolysin pore. The pore was constructed in vacuum, and restraints and implicit solvent with a low dielectric constant used to simulate the membrane environment. The control parameters for the multiple steered molecular dynamics simulations needed to obtain the potentials of mean force of translocation, pulling speed and force constant, were determined and tested with simulations of DNA translocation using the wild type and two different mutant pores, which were characterized by their translocation PMF, hydrogen bonding pattern, and pore diameter variation through the simulations.

The results are promising and show that the model is stable and respond adequately to the changes in the pore. In the next step, we will integrate in the pore the functionalization of the cysteines, which can be done experimentally by using water-soluble sulfydryl-specific reagents, and are expected to improve control over the DNA translocation speed.

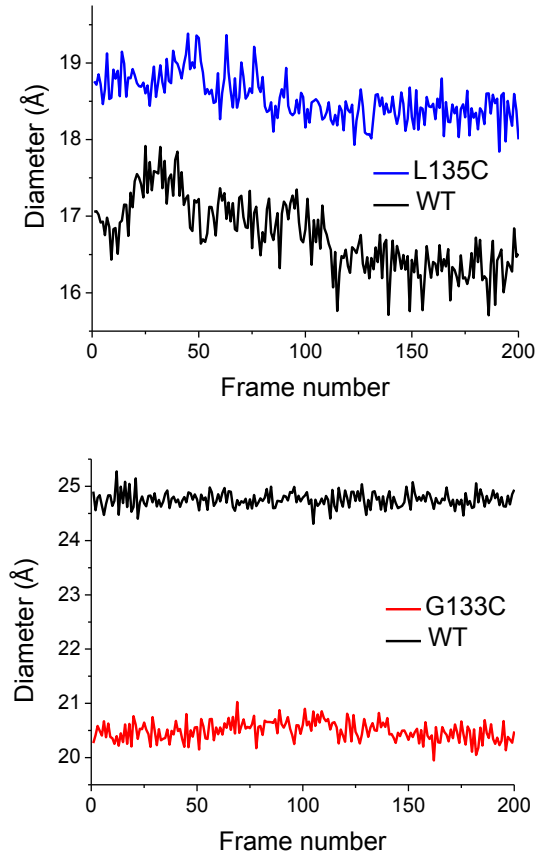

Figure 5. Average of diameter measurements in 135 and 133 positions during the translocation simulations. At the top, the measurements are all taken at position 135, while at the bottom all measurements are made at position 133.

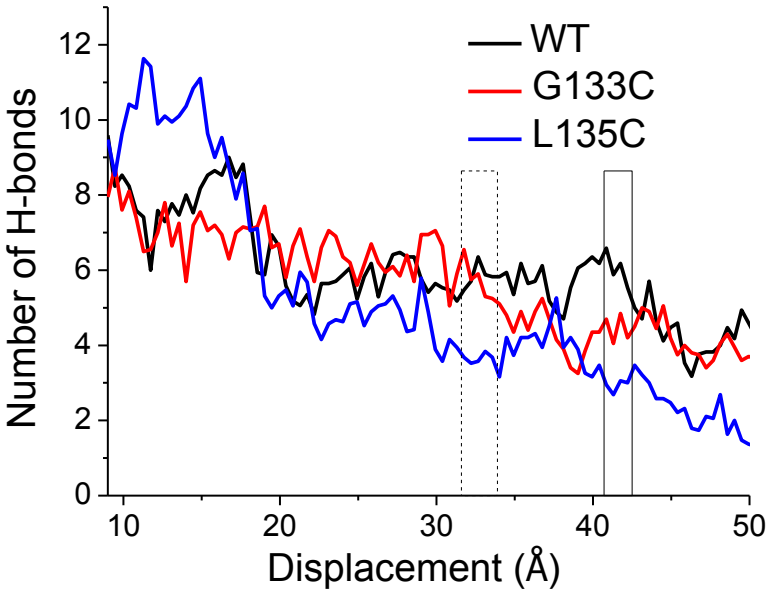

Figure 6. Total number of hydrogen bonds between 5 poly(dA) and α-hemolysin interior amino acids side chains, as a function of the displacement of the pulled atom. The dashed mark shows the 135 position and the solid mark shows the 133 position.

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5. CONCLUSÃO

Pode-se obter por meio de software de química computacional, a análise da alteração causada mutações realizadas na estrutura proteica do nanoporo da α-hemolisina, no diâmetro do poro, o que interferiu diretamente variação da energia livre que ocorre com a translocação da fita simples de DNA pelo interior do nanoporo. As mutantes escolhidas, G133C e L135C, juntamente com a forma nativa, foram empregadas em todos os cálculos de parametrização do sistema e na avaliação da variação de energia livre.

Determinaram-se os parâmetros ideais para a realização da dinâmica molecular com o sistema DNA – α-hemolisina em solvente implícito. Estes parâmetros estabelecidos foram os de velocidade igual a 1Å ps^{-1} e de constante de força igual a 100 kcal mol^{-1} $Å^{-2}$ que foram aplicados ao sistema usando a relação de Jarzynski.

Por meio da relação de Jarzynski, obteve-se a variação de energia livre, observando que a mutante G135C apresenta uma maior interação com a fita de DNA, além de um número aumentado de ligações de hidrogênio entre o DNA e o Interior do poro. Notou-se também que o diâmetro do nanoporo submetido à dinâmica molecular não teve grandes variações, oq eu mostra a efetividade do sistema, mesmo que simplificado.

6. PERSPECTIVAS

Com esses dados podemos obter a determinação dos parâmetros preliminares para posteriores alterações no nanoporo da α-hemolisina usando reagentes sulfidrila-específicos hidrossolúveis, os quais irão funcionalizar as cisteínas ligando-se a estas, alterando o perfil eletrostático do interior do nanoporo e a sua estrutura.

Sendo assim, temos como perspectivas de continuação deste trabalho, a parametrização das moléculas funcionalizadas para a análise da energia livre do sistema e melhoramento da parte experimental do sequenciamento por nanoporo de α-hemolisina.

7. ANEXOS

7.1. NORMAS DA REVISTA

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7.2. RESUMOS PUBLICADOS EM EVENTOS

7.2.1. SPECIFIC ANION EFFECT ON DNA TRANSLOCATION THROUGH PROTEIN NANOPORE publicado em **XXVI Reunião das Sociedades Brasileiras de Biologia Experimental** (2011).

ResumoID: 181-1

SPECIFIC ANION EFFECT ON DNA TRANSLOCATION THROUGH PROTEIN NANOPORE

BRITO, A.M.S.; MACHADO, D. C. ; RODRIGUES, C. G. ; KRASILNIKOV, O. V.
Departamento de Biofísica e Radiobiologia, UFPE

Keywords: alfa-hemolysin, DNA sequencing, Hofmeister, protein nanopore

Objectives:

In all currently used methodologies nucleic acids need to be marked and/or amplified before sequencing. The use of nanopore technology (that is believed to be a base of a new generation sequencers) does not demand amplification and/or labeling. Unitary α-hemolysin nanopore in planar lipid bilayers is supposed to be a key element of such sequencers where the "reading" of the nucleotide sequence will occur during electrophoretically driving translocation of single-stranded DNA (ssDNA) through the pore (Nanoscale, 2:468-483, 2010). However, the extremely high speed of ssDNA-translocation prevents a secure identification of nucleotides by this type of sequencer. The main objective of this study was to evaluate the influence of different anions on translocation speed of ssDNA molecules.

Methods and Results:

Planar lipid bilayer membranes of 40 pF capacitance were formed by the lipid monolayer apposition technique (Proc Nat Acad Sci USA, 69:3561-3566, 1972), using 1,2-diphytanoyl-sn-glycero-3-phosphocholine. *S. aureus* α-hemolysin was added from the cis side of the membrane in a concentration sufficient to form unitary protein nanopore in planar lipid membranes. The experiments were carried out at voltage clamp at room temperature of 25±1•C. The membrane-bathing aqueous solution contained 4 M KCl or KF (both 99.99% purity) in 5 mM Tris-citric acid buffer (pH 7.5). Poly-dA 50b ssDNA was used as a probe added in the cis compartment of the experimental chamber. As expected, the residence time (τoff) of the DNA inside of the pore was found to decrease with voltage. We have established the values of τoff depended strongly on the salt species demonstrating the specific (Hofmeister) effect. τoff was found to be significantly larger (22±2 times) in KF solution in compare with KCl. So a simple change in ion composition leads to considerable, more than one order of magnitude, improving the detection and analysis of nucleic acids.

Conclusions:

Modification of ion composition is the simple maneuvers considerably improves the resolution of kinetic parameters of DNA/α-hemolysin nanopore interaction and could be helpful for developing of nanopore based new generation DNA sequencers.

Financial Support: CNPq, FACEPE, INAMI

7.2.2. THE ENGINEERING OF PROTEIN-BASED NANOPORE FOR NUCLEOTIDE DETECTION publicado em **São Paulo School of Advanced Sciences** (2012)

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São Paulo School of Advanced Science
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THE ENGINEERING OF PROTEIN-BASED NANOPORE FOR NUCLEOTIDE DETECTION

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Biosensor is a sensor whose recognition element is a biological or biologically derived material. Recently, stochastic sensing properties made possible the development of a new category of sensors – stochastic biosensors. The ionic channel (nanopore) formed by alpha-hemolysin produced by Staphylococcus aureus is being used to detect several reduced-size analytes, being considered a promising stochastic biosensor. This biosensor is based on construction of a thin lipidic bilayer, in which the alpha-hemolisin nanopore (αHL) is incorporated. This membrane separates the aqueous ionic solution on two compartments, connected electrically only by the nanopore. A transmembrane voltage applied makes the charged solutes (dissociated ions) to flowing through nanopore generating ionic current. Besides, the nanopore has the capacity of "self-reposition", where a new molecule can only get inside after the other has left, providing identification of individual molecule. Unfortunately, there are some limitations on detecting some analytes, mainly the charged ones, specifically DNA, because they translocation too fast in the nanopore, difficulty nucleotide identification. To render these analytes detection more specific, we can change the protein structure chemically or genetically. In our work, we propose the construction of mutant proteins by site-directed mutation, by the cysteine-scanning technique, in which we change a pre-existing amino acid for a cysteine. Through this technique, we can use MTS reagents (methanethylsulfonate) to reduce sulfhydryl groups, which are present in cysteine. Depending on the used reagent, there are positive or negative charges present inside the nanopore. This strategy can render analytes detection to be more specific. Besides measurements of ionic currents though alpha-hemolysin mutants, molecular modeling techniques are being applied as tools to explore and optimize chemical interactions between the biosensor and analytes. Molecular docking and molecular simulations are the major procedure used to address such interactions.

Keywords: biosensor; alpha-hemolysin; computational modeling.

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Hofmeister Effect in Confined Spaces: Halogen Ions and Single Molecule Detection

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ABSTRACT Despite extensive research in the nanopore-sensing field, there is a paucity of experimental studies that investigate specific ion effects in confined spaces, such as in nanopores. Here, the effect of halogen anions on a simple bimolecular complexation reaction between monodisperse poly(ethylene glycol) (PEG) and α-hemolysin nanoscale pores have been investigated at the single-molecule level. The anions track the Hofmeister ranking according to their influence upon the on-rate constant. An inverse relationship was demonstrated for the off-rate and the solubility of PEG. The difference among anions spans several hundredfold. Halogen anions play a very significant role in the interaction of PEG with nanopores although, unlike K^+, they do not bind to PEG. The specific effect appears dominated by a hydration-dehydration process where ions and PEG compete for water. Our findings provide what we believe to be novel insights into physicochemical mechanisms involved in single-molecule interactions with nanopores and are clearly relevant to more complicated chemical and biological processes involving a transient association of two or more molecules (e.g., reception, signal transduction, enzyme catalysis). It is anticipated that these findings will advance the development of devices with nanopore-based sensors for chemical and biological applications.

INTRODUCTION

Because ion-channel proteins are so finely tuned to respond to specific molecules, they serve as models for developing nanopore devices for biomolecular sensing. A single nanometer-scale pore formed by *Staphylococcus aureus* α-hemolysin (αHL) is one of the most promising biological structures for creating a single molecule detector and analyzer. However, further development of these strategies is impeded by several technological and scientific problems, including difficulties in stabilizing a nanopore-membrane complex over a period of time and insufficient understanding of physics that regulate the rate at which molecules enter into nanocavities and the energy of their interactions.

The salting-out effect is a very general phenomenon, in which the solubility of a solute in water is decreased when electrolyte is added. It occurs in cultured microorganisms, in aqueous dispersions of macromolecules and amino acids, in self-assembled amphiphilic structures, in the surface tension of water, and even simple gas molecules (1–8). Cations and anions affect salting-out processes with widely varied effectiveness. The ordering of ions in terms of their effectiveness is known as the Hofmeister series (9–11). In recent years, there has been an explosion in research articles tied to the Hofmeister series (11–14); however, despite this increase in attention, a molecular-level understanding of the Hofmeister series is still lacking. Moreover, to the best of our knowledge there are only four isolated publications related to nanopores: the influence of Hofmeister anion series on αHL channel formation in lipid bilayers (15); the anion-dependent gating of roflamycoin ion channels (16); and the specific guest-host complexation of the cyclic carbohydrate, γ-cyclodextrin, with adamantane carboxylate inside the pore of αHL channels (17); and the influence of alkali chlorides on αHL channel conductance (18).

Our recent studies with poly(ethylene glycol) (PEG) as an analyte (19,20) demonstrated that an increase in KCl concentration from 1 M to 4 M, leads to high-resolution recording of PEG/αHL nanopore interactions. The effect of salt concentration is linked with an ~100-fold increase in the on- and off-rate constants of the process (21–24). It was suggested that salting-out was responsible for the change in the on-rate constant (22), and consequently for changes in the transition rate and the detection limit.

The goal of this study was:

1. To investigate the influence of halides on a simple bimolecular complexation reaction between monodisperse poly(ethylene glycol) and α-hemolysin nanoscale pores;
2. To reveal whether halogen anions have a specific effect on complex formation; and
3. To disclose the possible mechanism of action.

To achieve this and to improve the sensitivity of nanopore sensors, we performed systematic studies of the kinetics of PEG/αHL channel interactions to examine the effect of halogen anions. The key finding is that the type of halogen anion strongly influenced PEG/αHL nanopore interactions. Remarkably, both on-rates and off-rates were affected. The anions track the direct and inverse Hofmeister series for the on-rate, and the off-rate and the solubility of PEG, respectively. The difference among anions spans several hundredfold. The specific effect appears dominated by hydration-dehydration processes where anions and PEG compete for water.

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MATERIALS AND METHODS

Wild-type *S. aureus* α-hemolysin (αHL) was purchased from Calbiochem (Madison, WI). 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC) was purchased from Avanti Polar Lipids (Alabaster, AL). High quality (>99.99%) halides (KCl, KF, KBr, and KI) were purchased from Sigma (St. Louis, MO). Monodisperse poly(ethylene glycol), (molecular mass = 1294 g/mol; PEG), was purchased from Polypure (Oslo, Norway). The 2-amino-2-hydroxymethyl-1,3-propanediol (TRIS) and citric acid were from Schwarz/Mann Biotech (Cleveland, OH) and Fluka (Buchs, Switzerland), respectively.

Solvent-free planar bilayer lipid membranes, with a capacitance of 40 pF, were formed by the lipid monolayer apposition technique, using DPhPC in hexane (J. T. Baker, Phillipsburg, NJ) at 25 ± 1°C. Because it was recently shown that the elevated KCl concentrations considerably improve single molecule identification by unitary protein nanopores (19,20,22), in this study membrane-bathing solutions contained 4 M halide in 5 mM TRIS adjusted to pH 7.5 with citric acid.

PEG1294 was used as a representative analyte and added to the *trans* compartment of the experimental chamber. αHL was added from the *cis* side of the membrane in a concentration sufficient to form unitary protein nanopores in planar lipid membrane. If not mentioned otherwise, the applied potential was 40 mV. A positive current is defined by cation flow from *trans* to *cis*. Single αHL nanopore incorporation and measurement of molecular signature parameters (mean duration and amplitude of the blockage), transition rate, and kinetic constants of the PEG-nanopore interactions were done essentially as described by Rodrigues et al. (22). In short, the on-rate constant, k_{on}, was defined as $1/(C_{PEG} \times \tau_{on})$. The characteristic time, τ_{on}, was obtained from the collected time intervals between the end of one blockade event and the onset of the next. The off-rate constant, k_{off}, was defined as $1/\tau_{off}$, where τ_{off} is the characteristic time of PEG staying in the pore. The constant of PEG-αHL pore complex formation, K_f, was calculated by using the association (on-) k_{on} and dissociation (off-) k_{off} rate constants as k_{on}/k_{off}.

Experiments were done using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) in voltage-clamp mode. Membrane potential was maintained using Ag/AgCl electrodes in 3 M KCl 2% agarose bridges assembled within standard 200-μL pipette tips. Currents were filtered by a low-pass eight-pole Bessel filter (Model 9002; Frequency Devices, Haverhill, MA) at 15 kHz and directly saved into computer memory with a sampling frequency of 50–250 kHz. Experiments employed dilute polymer solutions. The greatest PEG concentration used in bilayer experiments was well below the overlap concentration (~13%) (25) and solubility for this PEG (see Results and Discussion). The limit of PEG solubility was estimated by the modified cloud-point method as described by Rodrigues et al. (22). Inaccuracy was <1%.

RESULTS AND DISCUSSION

Conductance

First, we examined the conductance of single αHL channels surrounded by 4 M solutions of different halides. At the chosen pH (7.5), the channel was in a high conductance state at all transmembrane potentials from −200 mV to +200 mV in all solutions. Conductance-voltage curves of αHL channels in KF, KBr, and KI solutions were found to be slightly asymmetric (data not shown), similar to that in KCl (26). Such behavior probably results from the asymmetry in charge distribution between the channel openings and in the channel structure itself. The channel conductance was considerably higher in solutions of KCl, KBr, and KI compared with KF solution (more than one-and-a-half times) (Fig. 1). This finding appears to be in accordance with ionic conductivities

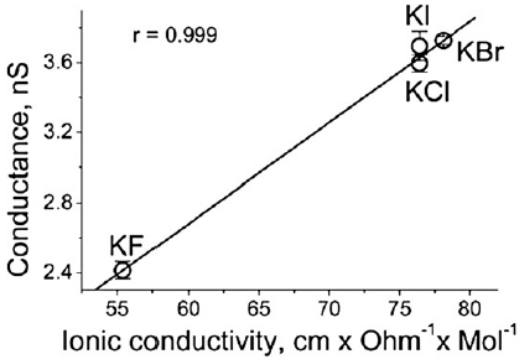

FIGURE 1 The correlation between the ionic conductivity of halogens and the αHL channel conductance (extrapolated to zero voltage) in 4 M halide solutions. Values of αHL channel conductance at zero voltage were obtained by fitting the experimental data points of *G/V* curves (obtained in the range of ±200 mV) with second-order polynomial function. (*Data points*) Means from at least three separate experiments ± SD. (*Line*) First-order regression fit of the data. The correlation coefficient is shown in the figure.

of halides (27) because a strong correlation between ionic conductivity of halogen anions and ion channel conductance was established (Fig. 1).

This result is consistent with earlier data for the channel obtained at low salt concentrations (28). In addition, we found that the ability of αHL to form ion channels was influenced considerably by the halogen ion species. It was highest in KCl (~10 pM of αHL is sufficient to form a single channel in the planar lipid bilayer) and smallest in KF solution (where up to 1 nM of αHL must be added). In KBr- and KI-solutions, the channel-forming activity was of intermediate magnitude. The result with KCl, KBr, and KI is consistent with earlier observations that the rate of αHL channel formation is inversely proportional to the size of the anions (15). However, the small channel-forming activity in the presence of KF in the bathing solution was unexpected. This influence of F^- needs further study.

Integral effect

Stable conductance of single αHL channels in all 4 M halide solutions was a prerequisite to using the channel as a sensor element. Using PEG1294 as an analyte, we found that the PEG effect is strongly influenced by anion type, and is voltage- and dose-dependent (Fig. 2). The maximal blocking effect was observed at 40 ± 10 mV. Such behavior is consistent with the findings of Rodrigues et al. (22) and indicates that molecules of nonionic PEG possess an acquired positive charge due to complex formation with K^+ in all halide solutions.

Single molecule events

As expected, high-resolution recordings of PEG/αHL interactions in the presence of different halides revealed a huge

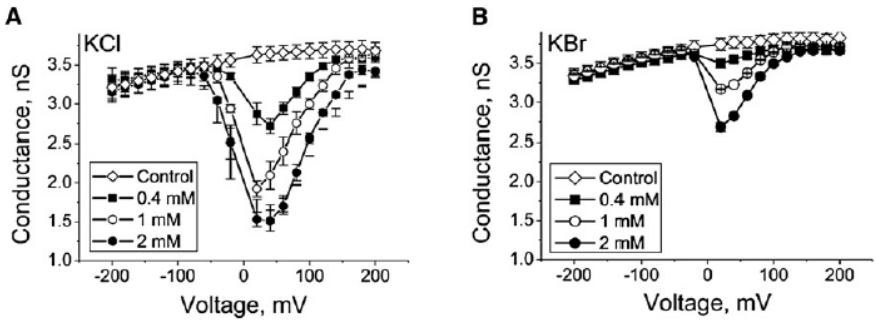

FIGURE 2 Effect of *trans*-side addition of PEG1294 to membrane-bathing solutions on channel conductance. (*A*) 4 M KCl; (*B*) 4 M KBr. The result of at least three independent experiments (mean ± SE) is presented in each case.

difference in the frequency and duration of well-defined current blockades (Fig. 3). Results obtained in KCl, KBr, and KI solutions indicate that the frequency of events is high in the presence of KCl, lower in the presence of KBr, and lowest in 4 M KI. The apparently lower frequency of PEG blockages in KF solution than in KI is delusive because there is a 40,000-fold difference in PEG concentration. It was not possible to use the same PEG concentration with KF-solutions due to much larger on-rate and much lower off-rate constants of the PEG/αHL interaction (see below). Interpolation of the KF results obtained at low PEG concentration (Fig. 3 *D*, 10 nM) to larger PEG concentrations projects much higher frequency values than seen in the presence of any other halides.

Rate constants of PEG/αHL channel interaction

To determine the reason for this anion-specific effect, we analyzed the rate constants of the process by essentially the same method as described recently by Rodrigues et al. (22). We have demonstrated that the on-rate constant (measured at an optimal 40 mV) is strikingly dependent on type of halogen (Fig. 4 *A*, *solid line*). The largest on-rate constant was observed with KF in the bath and the smallest in the presence of KI. The difference among on-rate values exceeded two orders of magnitude. The effect of anions follows the Hofmeister series: $F^- > Cl^- > Br^- > I$. The influence of anions on the off-rate constant follows the reverse Hofmeister series (Fig. 4 *A*, *dashed line*). The difference among off-rate values exceeded 20-fold, but was smaller than the difference among on-rate constant values. As a result, the formation constant of the PEG/αHL channel interaction follows the Hofmeister series (Fig. 4 *B*). Voltage dependencies of the kinetic constants in KF^-, KBr^-, and KI^- solution resemble those in KCl that we published recently (22). The off- and on-rate constants show turnover behavior with differences up to four orders of magnitude (data not shown). Detailed analysis of the effects of transmembrane voltage on dynamics of PEG-αHL channel interactions deserves careful study. These results will be included in a future work.

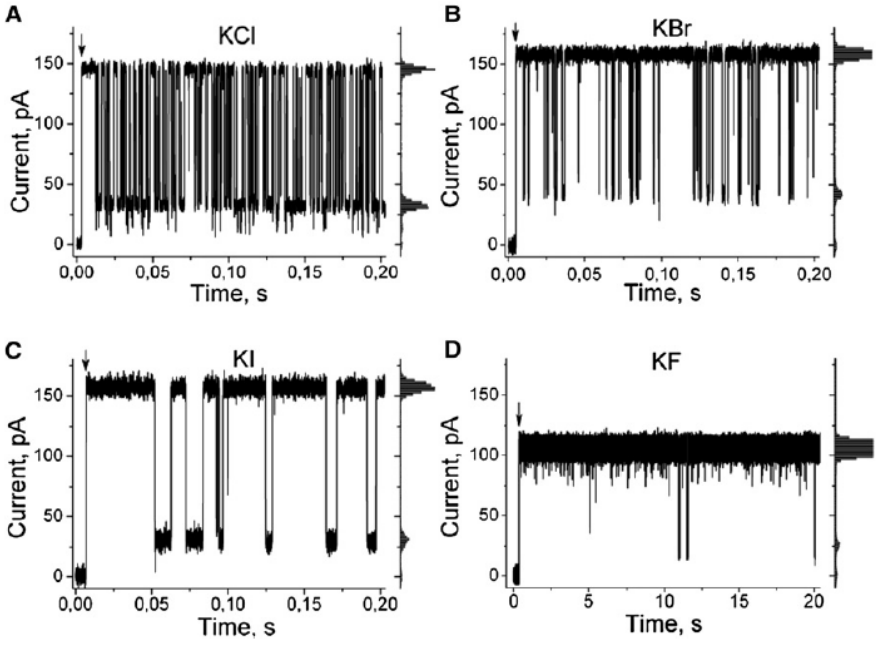

FIGURE 3 Typical traces of the ion current through single αHL channels in the presence of PEG1294 in the bath solution at a time resolution of 0.1 ms. (*Arrows*) Voltage shift from zero to 40 mV. Halide concentration was 4 M. Concentration of PEG was 400 μM (for *A–C*) and 10 nM (for panel *D*). Note the change in the timescale and PEG concentration from panels *A–D*. The respective all-point histograms are shown at the right of each record and used to give the mean value of blockage amplitudes. The effectiveness of such inhibition was very similar for all halides and comprised 73–80% of the maximal current.

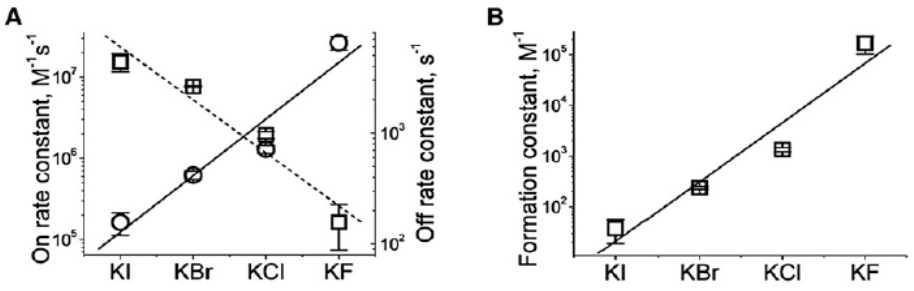

FIGURE 4 Specific ionic effect on the PEG1294/αHL pore interaction. The average on-rate (○, *solid line*), off-rate (□, *dashed line*) (*A*), and PEG/αHL pore complex formation constant (*B*) in 4 M solutions of different halides. (*Data points*) Means (± SD) of at least three single protein nanopores reconstituted in separate experiments with >5000 events as in Fig. 3. (*Lines*) Guide for the eye. The data presented in panel *A* were used to build the dependence shown in panel *B*.

PEG solubility in halide solutions

Salting-out was shown recently to affect the dependence of the on-rate upon KCl concentration (22). Therefore, we decided to verify whether salting-out is also responsible for the specific anion effects demonstrated above. The solubility of PEG in halide solutions was examined. The type of anion was found to exert a strong influence on PEG solubility. PEG solubility in 4 M KF (19 ± 2 μM) was more than two-orders-of-magnitude smaller than in 4 M KCl (3840 ± 390 μM). On the contrary, PEG solubility in 4 M KBr or 4 M KI was so large that it was not possible to determine precisely. To overcome this difficulty and obtain numerical values for all anions, we the carried out experiments with mixed (KF/KCl, KBr/KCl, and KI/KCl) solutions, keeping the total concentration at 4 M (Fig. 5).

Increasing the molar fraction of KF decreases PEG solubility, whereas increasing KBr monotonously increases it. PEG solubility as a function of KI concentration showed an anomalous molar fraction effect. KI decreased the solubility of PEG at small molar fractions, and started to increase only at a KI molar fraction larger than 0.15 (Fig. 5 *A*, *inset*). The reason for this effect has yet to be discovered.

Because PEG solubility in 4 M KBr and 4 M KI was practically impossible to determine, the values obtained at 1:1 molar ratios KX/KCl were used to compare with the kinetic constants of PEG/αHL pore interactions. A strong negative correlation exists between the on-rate constant and solubility, indicating the direct involvement of salting-out in this effect of halogen ions (Fig. 6 *A*). A positive correlation was established between the off-rate constant and PEG solubility (Fig. 6 *B*), reinforcing our conclusion that PEG salting-out dominates the specific effects of halogen ions on the on- and off-rates of PEG/αHL channel interactions.

Transition rate and detection limit

The PEG effect on αHL channel conductance is dose-dependent (Fig. 2). Recent studies (22–24,29) demonstrated that the event frequency (transition rate, $1/\tau_{on}$) is linearly related to the concentration of molecules of PEGs, cyclodextrin derivatives, and amino acids in KCl and NaCl solutions, thus providing a basis for quantifying these molecules. Here we have demonstrated that it is true for PEG in all halide solutions. In all cases the transition rate was directly proportional to analyte concentration (PEG in this case) with a slope of ~1 (0.97 ± 0.06) (Fig. 7). This finding suggests that the partitioning of analyte into the αHL pore can be described by a first-order reaction. Assigning reasonably the detection limit as a background equivalent PEG concentration at 1-Hz event frequency, we have established that it equals 8.0, 2.0, 0.5, and 0.05 μM (PEG1294) at 4 M of KI, KBr, KCl, and KF, respectively (Fig. 7). It is evident that the sensitivity of αHL pores to PEG depends crucially on anion type and is more than two-orders-of-magnitude higher in KF than in KI solutions.

It is known that experimental parameters, including pH, temperature, ionic strength, and applied potential have considerable influence on the resolution and sensitivity of stochastic

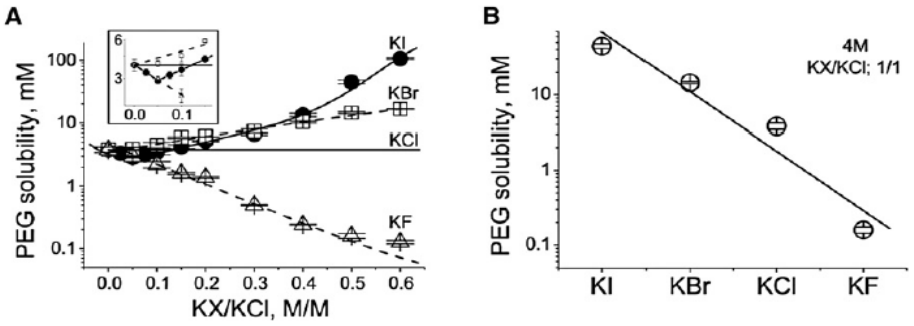

FIGURE 5 Solubility of PEG in 4 M halide solutions. (*A*) PEG solubility as a function of different molar fractions of KF, KBr, and KI in KCl solution. (*Inset*) The zone of the anomalous molar fraction effect for KI/KCl mixtures visualized at higher resolution. (*B*) Solubility of PEG at 1:1 (M/M) KX/KCl ratio, where *X* represents F^-, $Br-$, or I^-. (*Lines*) Guide for the eye. Total salt concentration was 4 M. (*Data points*) Means of 5–8 separate experiments ± SD.

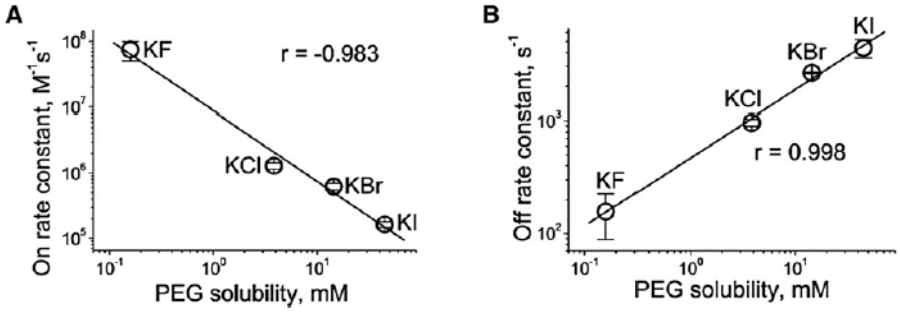

FIGURE 6 The on-rate (*A*) and off-rate (*B*) constants of PEG/αHL channel interaction as a function of PEG solubility. (*Data points*) Means of 5–8 separate experiments ± SD for solubility and at least three separate experiments for the rate constants. (*Lines*) First-order regression fits of the data. The correlation coefficient is shown in the figure.

detection. We have shown here that it could be significantly improved by simply using an appropriate type of electrolyte.

The role of hydration

In this study, halides were used at the same high (4 M) concentration where the difference in electrostatic interactions between analyte molecules and nanopore channels is significantly limited. However, a huge difference in kinetic constants of PEG/αHL channel interactions was established, indicating the specific ion effect (Hofmeister) in confined spaces such as those of protein nanopores. The competition between ions and analyte for water is frequently noted as one possible reason for Hofmeister effects. To verify that this competition is responsible for the observed effect of halides on PEG/αHL channel interactions, a correlation analysis between the rate-constants and anion hydration was performed. Very strong correlations between the rate constants of PEG/αHL channel interaction and the hydration enthalpy of anions were demonstrated (Fig. 8). Higher hydration of the anion resulted in a higher probability of the interaction and the stronger stability of the complex.

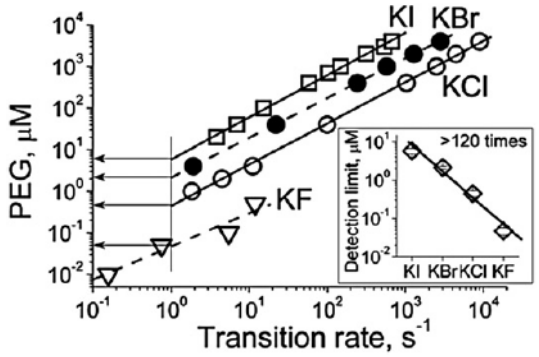

FIGURE 7 Transition rate and PEG concentration. The concentration of PEG1294 as a function of the transition rate in different halide solutions. (*Lines*) First-order regression fits of the data with a slope of 0.97 ± 0.06. (*Data points*) Means of at least three separate experiments. Standard deviations were omitted for clarity. (*Horizontal arrows*) Background equivalent PEG concentration at an event frequency of 1 Hz. (*Inset*) The background equivalent PEG concentration at different halide solutions. The number shown in the figure is the difference in values obtained in KI and KF solutions.

Higher hydration means that a large number of water molecules are bound to ions so that fewer of them are available to interact with cosolute (PEG, in this case). Because of this competition between ions and cosolute for water, it appears that ions exclude cosolutes from the bath to the protein nanopores which serve as harbors. Hence, the confined space presents more favorable conditions for PEG to compete with ions for water, indicating that the confined water is more accessible to PEG (and, probably, for other analytes (cosolute)). The existence of water that is accessible to nonionic analytes (confined water) accords with the presence of a hydration water layer at nanopore surfaces, where the presence of ions is severely restricted (30,31) and with remarkable decreases in the hydration number for ions confined in nanospaces (32).

Electroosmotic flow

All experiments presented in our study were made at high (4 M) salt concentrations. At first glance, a significant electroosmotic flow through αHL channel would not be expected under these conditions because it is known (26) that the cation-anion selectivity of αHL channel at high KCl concentrations is close to zero. This means that the channel is unselective and the transport number of cations and anions is equal to ~0.5. On the other hand, it seems reasonable to assume that for this condition (nonselective channel), the water transported per ion might be equal to that in the primary hydration sphere. Thereby one would expect that despite a similar transport number between cations and anions, the net water transport will not equal zero, because the hydration numbers of the anions (4.97, 2.87, 2.30, and 1.67 (27) for F^-, Cl^-, Br^-, and I^-, respectively) are significantly different from the hydration number for K^+ (3.33).

Moreover, the anions rank in Hofmeister series according to their hydration number. If this parameter dominated the kinetics of PEG/αHL channel interactions, the on-rate constant might be largest in KI and the lowest in KF solution, that is, exactly the opposite to the established dependence (Fig. 4 *A*). Ionic mobility is another important characteristic of ions that could influence the results. However, their ionic mobilities do not correspond to their

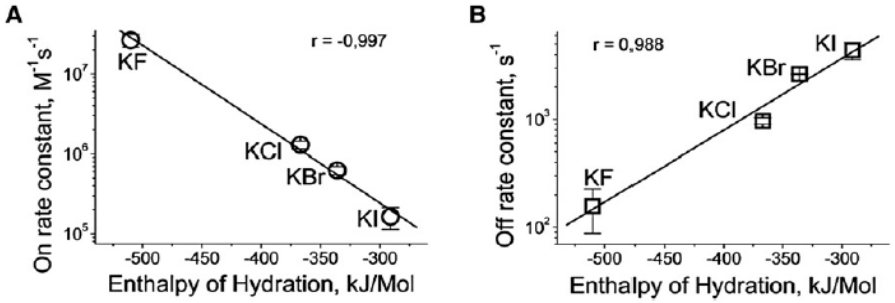

FIGURE 8 Rate constants of PEG/αHL channel interactions in halide solutions as function of hydration enthalpy of anions. Enthalpy values were taken from Marcus (27). The correlation coefficient is shown in the figure.

rank in the Hofmeister series: F^- has the lowest mobility (55.4 cm^2 Ohm^{-1} Mol^{-1}), whereas the mobilities of other three anions are close to each other (76.4–78.1 cm^2 Ohm^{-1} Mol^{-1}) (27). The foregoing reasons permit us to conclude that in 4 M potassium halide solutions, electroosmotic flow does not significantly affect the observed dynamics, if at all.

CONCLUDING REMARKS

In this work, we systematically investigated the interaction of the nonionic polymer, PEG, with a protein pore formed by αHL in planar lipid bilayers in solutions of different halides. We found that the type of anion has very strong influence on the rate constants of the process (the difference reaches several hundredfold). As a consequence, the transition rate and the detection limit of the nanopore-based sensor were correspondingly changed. All probed anions follow the Hofmeister ranking according to their influence on the on-rate constant ($F^- > Cl^- > Br^- > I^-$). An inverse relationship was demonstrated for the off-rate and the solubility of the analyte ($F^- < Cl^- < Br^- < I^-$). Therefore, a salting-out phenomenon is responsible for the anion-induced effect on single molecule detection with solitary protein nanopores. The specific effect appears dominated by hydration-dehydration (chemical) processes where ions and cosolutes compete for water.

We have shown that halogen ions play a very significant role in creating conditions for polymers to interact with the nanopores although K^+ (not anions) binds with PEG (22,33,34). Anion influence may provide an explanation for why the residence time for PEG in KCl solution is ~100-fold longer than expected from a theoretical model based on an electrostatic (Poisson-Nernst-Planck) approach (35). Hereby our findings provide what we believe to be previously undescribed insights into physicochemical mechanisms involved in single-molecule interactions with nanopores. The results will advance the development of devices with nanopore-based sensors for chemical and biological applications. Moreover, though our study deals with a simple first-order kinetic complexation reaction, our results are clearly relevant for more complicated chemical and biological processes, which involve a transient association of two or more molecules (e.g., reception, signal transduction, enzyme catalysis) where the hydration-dehydration reactions occur.

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