



UNIVERSIDADE ESTADUAL PAULISTA
“JÚLIO DE MESQUITA FILHO”

Faculdade de Odontologia de Araçatuba

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Relatório Final de Pós-Doutorado

Programa de Pós-Doutorado na UNESP - 12/2022 (PD-III: Sem financiamento)

**DESENVOLVIMENTO DE MEMBRANAS BIOATIVAS DE LÁTEX PARA
REGENERAÇÃO ÓSSEA. AVALIAÇÃO DE PROPRIEDADES MECÂNICAS E
BIOCOMPATIBILIDADE *IN VITRO***

Supervisor: Professor Associado Francisley Ávila Souza

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BIOCOMPATIBILIDADE *IN VITRO***

Relatório final apresentado à Faculdade de Odontologia do Campus de Araçatuba – Universidade Estadual Paulista “Júlio de Mesquita Filho” - UNESP, realizado durante o curso de PÓS-DOCTORADO (Grande Área: CIÊNCIAS DA SAÚDE, Área: ODONTOLOGIA, Subárea: CIRURGIA BUCO-MAXILO-FACIAL).

Supervisor: Prof. Dr. Francisley Ávila Souza

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RESUMO

A regeneração óssea guiada melhora o crescimento ósseo ao utilizar membranas para evitar a invasão de tecidos moles em defeitos ósseos. O látex, conhecido por sua biocompatibilidade e resistência mecânica, é um material promissor. A incorporação de substâncias bioativas, como o β -fosfato tricálcico (β -TCF), que oferece propriedades osteocondutoras e osteoindutoras, aumenta ainda mais a eficácia das membranas de látex na regeneração óssea. Este estudo teve como objetivo avaliar a biocompatibilidade e citotoxicidade de membranas de látex (ML) revestidas com β -TCF em escalas micro (ML- μ TCF) e nano (ML-nTCF), utilizando fibroblastos gengivais humanos (FGH-1). Além disso, membranas comerciais não reabsorvíveis feitas de politetrafluoroetileno denso e expandido (dPTFE e ePTFE) foram incluídas como controles positivos para comparação. As membranas foram recortadas no diâmetro de 10 mm, e a investigação da topografia de superfície foi realizada por meio de Microscopia Eletrônica de Varredura (MEV), medições de ângulo de contato e espectroscopia Raman. Para a contagem de células e análise de viabilidade, as membranas foram semeadas com densidade de 30.000 células/cm² e incubadas em triplicata por 24, 48 e 120 horas, com o poliestireno (PS) como controle negativo. A contagem de células foi conduzida utilizando o sistema NucleoCounter, e ensaios de atividade da lactato desidrogenase (LDH) foram realizados para avaliar a citotoxicidade. A análise dos dados foi realizada no GraphPad Prisma, com nível de significância de 0,05 para todos os testes. A análise por MEV confirmou a deposição de β -TCF, resultando em maior rugosidade superficial e densidade de partículas em ambas ML- μ TCF e ML-nTCF. Por outro lado, as membranas de PTFE exibiram estruturas mais lisas e porosas. As medições de ângulo de contato revelaram que apenas ML- μ TCF apresentaram comportamento hidrofílico, enquanto as membranas de PTFE foram totalmente hidrofóbicas, como esperado devido à composição do material. A análise por espectroscopia Raman confirmou a predominância de apatita carbonatada e látex em ambas as membranas experimentais. Ensaios de contagem de células revelaram que as ML-nTCF melhoraram significativamente a adesão e proliferação celular em comparação ao PS. Em 24 e 48 horas, ambas as membranas experimentais demonstraram a maior adesão inicial de células, indicando uma proliferação robusta, similar ao comportamento observado nas membranas de PTFE. Após 120 horas, todas as membranas apresentaram resultados semelhantes, com maior proliferação em comparação ao PS. Ensaios de atividade de LDH indicaram que as células se comportaram de maneira semelhante em todas as membranas, sem diferenças estatísticas observadas em qualquer momento avaliado. Por fim, a análise por MEV revelou a adesão e crescimento celular com morfologia fisiológica normal nas membranas. Este estudo demonstra que as membranas experimentais aumentam a adesão e proliferação celular, conforme evidenciado pelos ensaios de contagem de células e atividade de LDH. Essas propriedades tornam as ML- μ TCF e ML-nTCF promissoras para aplicações clínicas.

Palavras-Chave: Biomaterial, regeneração óssea guiada, modificação de superfície.

ABSTRACT

Guided bone regeneration enhances bone growth by using membranes to prevent soft tissue invasion into bone defects. Latex, known for its biocompatibility and mechanical strength, is a promising material. Incorporating bioactive substances like β -tricalcium phosphate (β -TCP), which offers osteoconductive and osteoinductive properties, further enhances the effectiveness of latex membranes for bone regeneration. This study aimed to assess the biocompatibility and cytotoxicity of latex membranes (LM) coated with β -TCP at micro (LM- μ TCP) and nano (LM-nTCP) scales, using human gingival fibroblasts. Additionally, commercially available non-resorbable membranes made of dense and expanded polytetrafluoroethylene (dPTFE and ePTFE) were included as positive controls for comparison. The membranes were punched to a diameter of up to 10mm and the surface morphology investigation were performed by using Scanning Electron Microscopy (SEM), contact angle measurements and Raman spectroscopy. For cell counting and viability analysis, membranes were seeded with cells at a density of 30,000 cells/ml and incubated in triplicate for 24, 48, and 120 hours, with polystyrene (PS) as the negative control. Cell counting was conducted using the NucleoCounter system, and lactate dehydrogenase (LDH) activity assays were performed to assess cytotoxicity. Data analysis was conducted using GraphPad Prism, with a significance level of 0.05 for all tests. SEM analysis confirmed the deposition of β -TCP on LM, resulting in increased surface roughness and particle density in both LM- μ TCP and LM-nTCP. Conversely, both PTFE membranes exhibited smoother and more porous structures. Contact angle measurements revealed that only the LM- μ TCP exhibited hydrophilic behavior, while the PTFE membranes were entirely hydrophobic, as expected, due to their material composition. Raman spectroscopy analysis confirmed the predominance of carbonated apatite and latex in both experimental membranes. Cell counting assays revealed that LM-nTCP significantly enhanced cell adhesion and proliferation compared to PS. At 24 and 48 hours, both experimental membranes demonstrated the highest initial cell attachment, indicating robust cell proliferation, similar to the behavior observed in the PTFE membranes. By 120 hours, all membranes showed similar results, with higher proliferation than PS. LDH activity assays indicated that cells behaved similarly across all membranes, with no statistical differences observed at any evaluated time point. And SEM analysis revealed cell attachment and growth with normal physiological morphology on the membranes. This study demonstrates that the experimental membranes enhance cell adhesion and proliferation, as evidenced by cell counting and LDH activity assays. These properties make LM- μ TCP and LM-nTCP promising for clinical applications.

Keywords: Biomaterial, guided bone regeneration, surface modification.

1. INTRODUÇÃO

A Regeneração Óssea Guiada (ROG) é uma técnica amplamente utilizada na reconstrução de defeitos ósseos, tendo como princípio o uso de membranas que atuam como barreiras físicas para evitar a invasão de tecidos moles no local da regeneração. Esse método tem se mostrado altamente eficaz, pois promove um ambiente controlado que favorece a formação de novo osso ao proteger o coágulo sanguíneo e inibir a presença de células osteoclásticas provenientes dos tecidos circundantes^{1,2}. Além de atuar como barreiras, essas membranas têm potencial para estimular processos regenerativos por meio do recrutamento celular e da indução de angiogênese³. Evidências clínicas reforçam o papel fundamental das membranas na melhoria da formação óssea, destacando sua importância em procedimentos de regeneração⁴.

Entre os materiais amplamente utilizados para a confecção dessas membranas estão as de politetrafluoroetileno (PTFE), que podem ser encontradas nas versões densa (dPTFE) e expandida (ePTFE). Reconhecidas por sua biocompatibilidade e resistência à infiltração microbiana, essas membranas têm sido preferidas em procedimentos de ROG e regeneração tecidual guiada (RTG)⁵⁻⁸. Membranas de ePTFE apresentam estrutura porosa que facilita a integração tecidual, enquanto dPTFE, com sua superfície mais lisa, reduz o risco de contaminação bacteriana quando exposto na cavidade oral⁹⁻¹². No entanto, sua natureza não reabsorvível exige remoção cirúrgica em um segundo procedimento, o que pode limitar sua aplicabilidade e aumentar os custos^{13,14}.

Paralelamente, novos materiais vêm sendo desenvolvidos para superar as limitações das membranas tradicionais, explorando características como biodegradabilidade, bioestímulo e custo acessível. O látex, por exemplo, desponta como uma alternativa viável devido à sua comprovada biocompatibilidade e versatilidade mecânica¹⁵. Membranas de látex (ML) têm demonstrado potencial em aplicações médicas, com destaque para sua capacidade de ajustar propriedades como porosidade, permitindo maior controle sobre integração tecidual e liberação de substâncias bioativas¹⁶. Embora a porosidade não seja um requisito para a ROG, ela pode melhorar os resultados regenerativos, dependendo das necessidades clínicas¹⁷.

O aprimoramento das propriedades das membranas também tem sido foco de estudos recentes, principalmente por meio da incorporação de substâncias bioativas. Entre elas, destaca-se o β -tricálcico-fosfato (β -TCF), um biomaterial amplamente utilizado devido às suas propriedades osteocondutoras e osteoindutoras¹⁸. Além de favorecer a regeneração óssea, o β -TCF apresenta uma estrutura microporosa que melhora a sobrevivência celular e estimula a vascularização, elementos cruciais para o sucesso da regeneração tecidual¹⁹⁻²². Estudos têm demonstrado que combinações de β -TCF com polímeros biocompatíveis, como ácido polilático-co-glicólico (PLGA) e policaprolactona (PCL), ampliam seu potencial, oferecendo suporte mecânico e promovendo a formação óssea^{22,23}.

Com base nesse contexto, o presente estudo avalia a biocompatibilidade e citotoxicidade de membranas de látex revestidas com β -TCF em escalas micro (ML- μ TCF) e nanométricas (ML-nTCF), utilizando fibroblastos gengivais humanos (FGH-1).

MATERIAIS E MÉTODOS

1.1. *Obtenção das Membranas Experimentais*

Este Projeto de Pesquisa integra uma Pesquisa Multicêntrica que propõe o desenvolvimento de membranas de látex com a incorporação de β -TCF nas escalas micro e nanométricas, no intuito de inovação tecnológica. A Pesquisa Multicêntrica será desenvolvida no Instituto de Química de Araraquara da Universidade Estadual Paulista (IQAr-UNESP), Faculdade de Odontologia de Araçatuba – UNESP, Faculdade Ciências Farmacêuticas de Araraquara – UNESP e Universidade de Gotemburgo. Membranas densas de PTFE (Cytoplast TM TXT-200, Osteogenics Biomedical Inc., TX, EUA) e expandidas (NeoGen™, Neoss Ltd., Harrogate, Reino Unido) serviram como materiais de controle positivo, selecionados com base em sua eficácia bem documentada demonstrada por pesquisas científicas anteriores.

1.1.1. *Síntese do β -TCF pelo método de secagem do sol-gel*

Os materiais para a síntese do β -TCF foram os reagentes de qualidade analítica: nitrato de cálcio $\text{Ca}(\text{NO}_3)_2 \cdot \text{H}_2\text{O}$ (PA-ACS) e ácido fosfórico H_3PO_4 85% (resfriado em banho de gelo), dissolvidos separadamente em álcool metílico CH_3OH (PA-ACS) sob agitação. A solução de ácido fosfórico foi adicionada à solução de nitrato de cálcio em banho de gelo permanecendo sob agitação (Figura 1). As concentrações dos reagentes foram escolhidas de modo a obter as razões molares Ca/P de 1,00; 1,65 e 1,50.

A solução obtida foi seca a 100°C até a evaporação do solvente, e o pó obtido foi fracionado e tratado a temperatura de 1200°C com uma taxa de aquecimento de $5^\circ\text{C}/\text{min}$ para a obtenção da fase de fosfato de cálcio. A identidade da fase foi confirmada através das fichas cristalográficas padrões da base de dados software Search Match®.

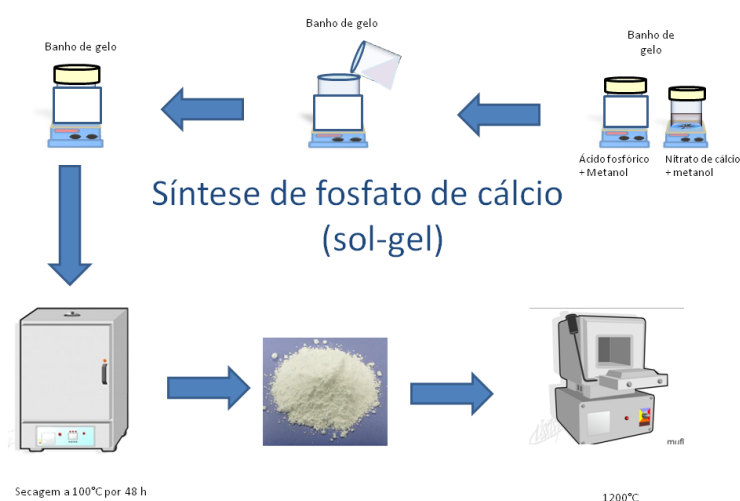


Figura 1. Fluxograma da síntese para a obtenção do fosfato de cálcio.

1.1.2. *Extração e preparação do LN*

O látex natural utilizado neste trabalho foi adquirido da BDF Comércio de Produtos Agrícolas LTDA, Guarantã - SP. Este látex foi obtido da mistura de dois clones: RRIM 600 e PB 235 (Lote:

01703/13). Depois da extração, o látex foi mantido em fase líquida através da adição de hidróxido de amônio corrigindo a acidez e estabilizando o meio ao pH igual a 10,2. Posteriormente, o polímero foi submetido à centrifugação de 8000g por 2 horas, com o objetivo de diminuir a quantidade de proteínas de alto peso molecular, responsáveis por reações de hipersensibilidade.

1.1.3. Incorporação das fases de fosfato de cálcio em membranas de LN

A fabricação das membranas consistiu em depositar 5mL de LN sobre a placa circular, mantendo-as em temperatura ambiente por 24h. Após a polimerização do látex foram adicionados sob a membrana 10mL de suspensão de β -TCF (10mg/mL) e mantidos em temperatura controlada de 50°C por 72h, para a absorção do fosfato de cálcio precipitado na superfície do látex (Figura 2).

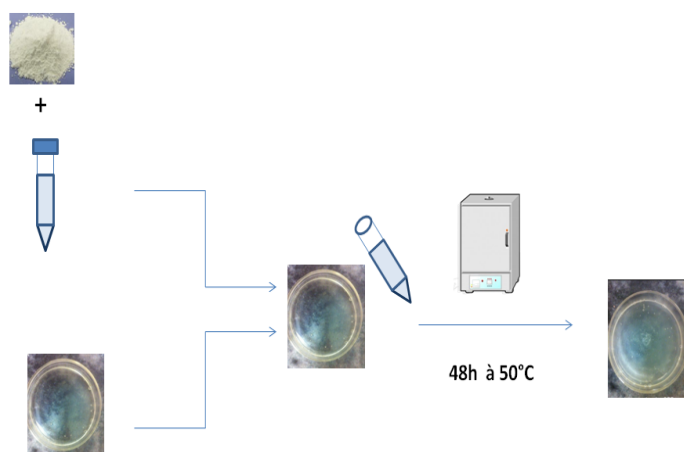


Figura 2. Fluxograma da confecção das membranas de LN e incorporação do β -TCF.

1.2. Caracterização topográfica

As membranas foram recortadas para obter um diâmetro de até 10 mm para a avaliação morfológica. As amostras foram montadas em suportes de alumínio usando fita de carbono condutiva e revestidas com uma camada de 10nm de ouro por meio de pulverização catódica. A microscopia eletrônica de varredura (MEV) foi realizada usando um Ultra 55 FEG SEM (Leo Electron Microscopy Ltd, Reino Unido) no modo de elétrons secundários com um detector in-lens, operado a uma voltagem de aceleração de 5kV, distância de trabalho de 5mm e tamanho de abertura de 30 μ m.

As medições do ângulo de contato estático foram realizadas com um software Biolin Scientific (Attension Theta Flow, SN TFW001594, 2023, Espoo, Finlândia) usando água deionizada. Apenas uma gota foi utilizada por superfície e pelo menos 3 amostras de cada membrana foram separadas especificamente para essa análise. Os valores médios foram calculados e tabulados para serem levados ao teste estatístico.

1.3. Espectroscopia Raman

A espectroscopia Raman foi empregada para investigar a composição das superfícies da membrana. Os espectros foram coletados em temperatura ambiente usando um detector de dispositivo de carga acoplada (CCD) resfriado a nitrogênio conectado a um espectrômetro Dilor XY 800 (Horiba, Jobin Yvon GmbH). O espectrômetro foi equipado com um laser Ar/Kr de 676 nm

operado a aproximadamente 20 mW, uma objetiva de 100×, grade de 600 sulcos/mm, distância focal de 300 mm e um tamanho de furo de 530 µm.

Os espectros brutos, truncados entre 200–1800 cm⁻¹, passaram por subtração de fluorescência de fundo ajustando uma função polinomial de sexta ordem usando o programa Background Correction para MATLAB R2013b (Mathworks Inc., Natick, MA). O eixo do número de onda foi ajustado de modo que os picos de ν1 PO₄³⁻ em todos os espectros correspondessem a aproximadamente 960 cm⁻¹. Os espectros corrigidos da linha de base foram então normalizados para a intensidade da banda de ν1 PO₄³⁻ usando Plot (<http://plot.micw.eu/>). Ajuste de curva, usando funções Gaussianas e Lorentzianas mistas, e quantificação de áreas integrais foram realizadas usando MagicPlot (www.magicplot.com).

1.4. Cultura de células

Uma linha celular de fibroblastos gengivais humanos primários (FGH-1, passagem 17, ATCC, EUA) foi cultivada em frascos de cultura de células (T75) em CO₂/ar humificado a 7% em meio de Eagle modificado por Dulbecco (Gibco, Manchester, Reino Unido) contendo 10% de soro fetal bovino aprovado pela UE inativado pelo calor (Gibco), 100 unidades/ml de penicilina, 100lg/ml de estreptomicina e 0,25lg/ml de Fungizone®, (antibiótico-antimicótico; Gibco). A temperatura foi fixada em 37°C e o meio de cultura foi trocado duas vezes por semana. Na confluência de 90-95%, as células foram lavadas com HBSS (solução salina balanceada de Hanks sem Mg²⁺ e Ca²⁺; Gibco), separadas usando 0,25% de tripsina em 1mM EDTA (Gibco) e divididas na proporção de 1:2.

1.4.1. Procedimento experimental

Noventa microlitros (µl) de suspensão celular contendo 3 x 10⁴ células/ml foram adicionadas a cada poço contendo as membranas, em triplicata, e incubados em uma placa de 48 poços por 24, 48 e 120 horas. O meio de cultura foi inicialmente trocado após 24 horas. Plástico tratado para cultura de tecidos (PS) serviu como controle.

Os números de células associadas às superfícies das membranas foram determinados usando um sistema NucleoCounter® (ChemoMetec, A/S, Allerød, Dinamarca). Após os períodos de incubação especificados, as membranas foram removidas e colocadas em um poço limpo. As células na superfície das membranas foram então tratadas com tampão de lise e tampão de estabilização (fornecidos com o sistema), carregadas em NucleoCasette™ pré-revestido com corante fluorescente de iodeto de propídio para coloração dos núcleos, e quantificadas no NucleoCounter®.

1.4.2. Viabilidade celular

A viabilidade celular foi determinada medindo a lactato desidrogenase (LDH), um marcador de lesão da membrana celular, no meio de cultura usando uma avaliação espectrofotométrica da conversão mediada por LDH de ácido pirúvico em ácido lactídeo (C-Laboratory, Sahlgrenska University Hospital, Gotemburgo, Suécia).

1.5. Análise estatística

Os experimentos foram repetidos pelo menos três vezes. A análise estatística foi baseada em Two-way ANOVA com correção para múltiplas comparações. As análises foram realizadas utilizando o software Prisma v10 (GraphPad Software, La Jolla, CA, EUA). O nível de significância foi definido como $p < 0,05$.

2. RESULTADOS

2.1. Padrões de Rugosidade da Superfície Avaliados por MEV

Para caracterizar a rugosidade da superfície de cada amostra, foi realizada análise por MEV. A distribuição das partículas de β -TCF sobre a superfície resultou em uma paisagem texturizada e granulada. Tanto as membranas ML- μ TCF (Figura 3) quanto ML-nTCF (Figura 4) apresentaram topografia superficial semelhante, com poros de formas irregulares e tamanhos variados, além de uma aparência de composição óssea mais evidente na ML-nTCF. As partículas em escala nanométrica mostraram-se densamente organizadas, preenchendo os espaços entre partículas maiores em escala micrométrica, destacando uma estrutura hierárquica no revestimento. Essa superfície altamente texturizada, com maior rugosidade e complexidade, pode favorecer interações celulares e a integração tecidual.

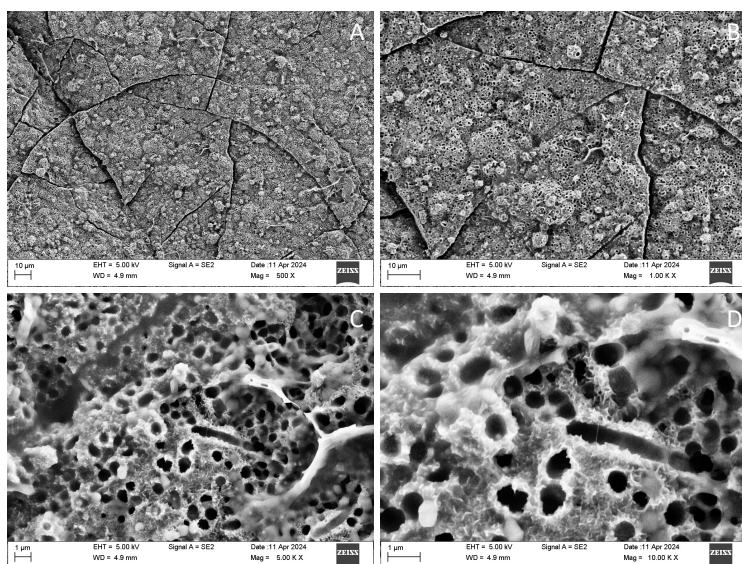


Figura 3. Padrões de rugosidade da superfície avaliados por MEV em ML- μ TCF. As descrições detalhadas das superfícies das membranas em diferentes aumentos 500x(A), 1000x (B), 5000x (C) e 10000x (D). Barras de escalas indicadas nas imagens.

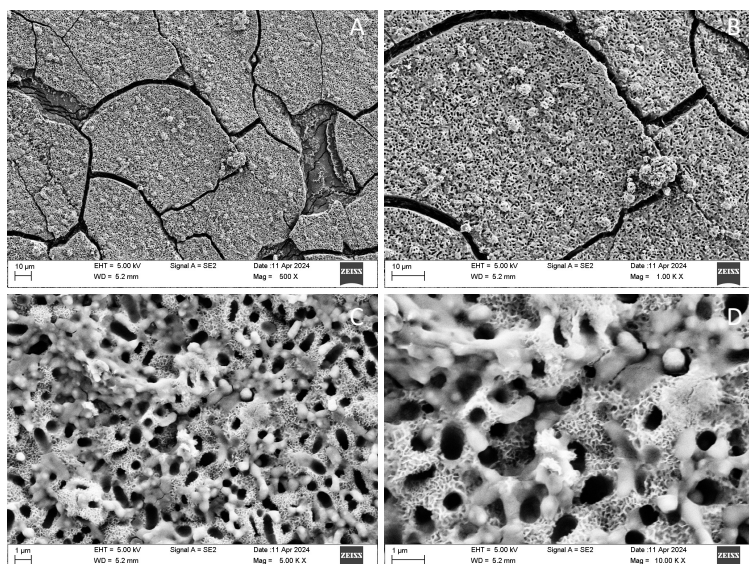


Figura 4. Padrões de rugosidade da superfície avaliados por MEV em ML-nTCF. As descrições detalhadas das superfícies das membranas em diferentes aumentos 500x(A), 1000x (B), 5000x (C) e 10000x (D). Barras de escalas indicadas nas imagens.

A membrana dPTFE (Figura 5) exibiu uma estrutura fibrosa com baixa porosidade; poros pequenos e compactados tornaram-se visíveis sob alta ampliação. Essa organização densa contribui para a resistência mecânica e impermeabilidade da membrana, tornando-a adequada para aplicações que requerem uma barreira robusta. Por outro lado, a membrana ePTFE (Figura 6) apresentou uma topografia marcadamente diferente, caracterizada por uma arquitetura aberta e porosa. A estrutura expandida do PTFE revelou uma rede de poros interconectados e fibras, criando uma aparência semelhante a uma malha esponjosa. Com poros maiores e irregulares, essa configuração oferece alta área superficial e permeabilidade, promovendo a troca de fluidos e infiltração celular, o que torna as membranas ePTFE ideais para aplicações que demandam maior permeabilidade e biocompatibilidade.

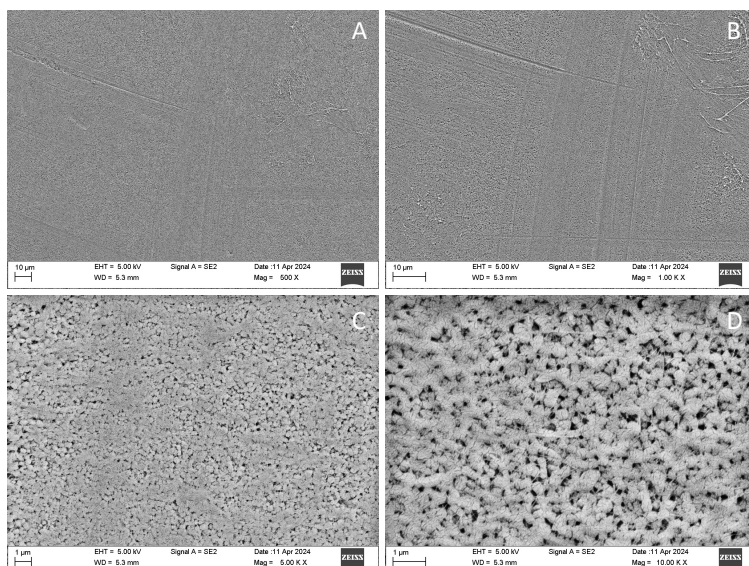


Figura 5. Padrões de rugosidade da superfície avaliados por MEV em membrana de dPTFE. As descrições detalhadas das superfícies das membranas em diferentes aumentos 500x(A), 1000x (B), 5000x (C) e 10000x (D). Barras de escalas indicadas nas imagens.

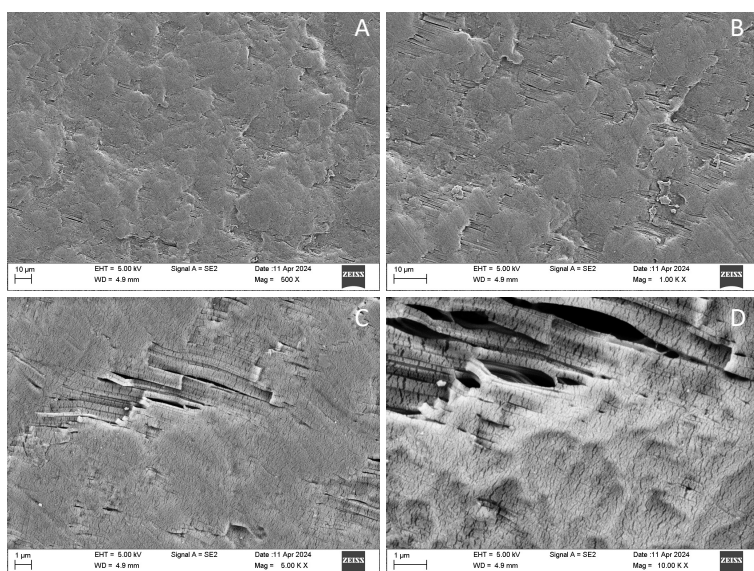


Figura 6. Padrões de rugosidade da superfície avaliados por MEV em membrana de ePTFE. As descrições detalhadas das superfícies das membranas em diferentes aumentos 500x(A), 1000x (B), 5000x (C) e 10000x (D). Barras de escalas indicadas nas imagens.

2.2. Medidas do ângulo de contato

Para avaliar a molhabilidade da superfície, foram realizadas medições do ângulo de contato. Imagens representativas das gotas de líquido sobre as superfícies das membranas estão apresentadas na Figura 7A, enquanto os valores correspondentes dos ângulos de contato estão ilustrados na Figura 7B. A membrana ML- μ TCF apresentou os menores valores de ângulo de contato ($<90^\circ$), indicando características de superfície hidrofílica. Em contrapartida, a ML-nTCF apresentou valores de ângulo de contato mais elevados em comparação à ML- μ TCF; no entanto, essa diferença entre os dois tipos de membranas experimentais não foi estatisticamente significativa.

As membranas dPTFE e ePTFE, de acordo com suas propriedades intrínsecas hidrofóbicas, exibiram ângulos de contato mais elevados, superiores a 90° . Diferenças estatisticamente significativas nos ângulos de contato foram observadas entre a ML- μ TCF e ambas as membranas de PTFE, reforçando a distinção entre as características de molhabilidade das membranas experimentais e comerciais.

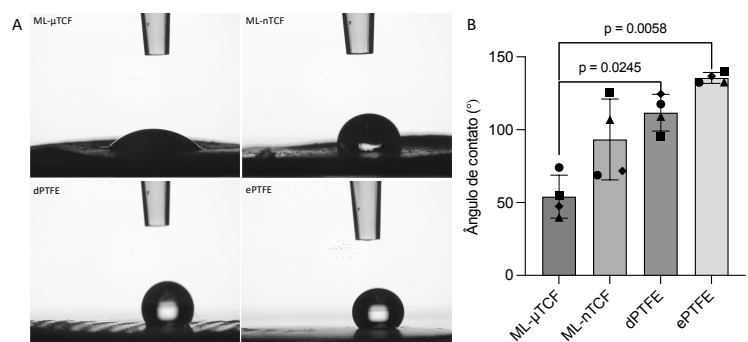


Figura 7. Medições de ângulo de contato indicando as características de molhabilidade das membranas analisadas. (A) Imagem representativa do ângulo de contato na superfície e (B) valores de ângulo de contato. Cada formato de ponto no gráfico representa os valores de ângulo de contato obtidos em experimentos individuais, apresentados com os valores médios e o desvio padrão (DP). A análise estatística foi realizada por meio de Two-way ANOVA, e os valores de p estão indicados no gráfico.

2.3. *Raman*

A espectroscopia Raman foi realizada para confirmar a fase do β -TCF depositado em ML. Nas membranas de látex natural (LN), as especificações Raman revelam picos específicos associados à estrutura do cis-1,4-poliisopreno, principal componente do látex natural. Os principais picos incluem a banda de estiramento C=C em torno de 1660 cm^{-1} e modos de flexão CH_2 e CH_3 proeminentes devido aos grupos metil e metileno ao longo da cadeia polimérica, próximos a 1440 cm^{-1} e 1375 cm^{-1} , respectivamente, conforme mostrado na Figura 8. Esses sinais são indicativos da estrutura de hidrocarbonetos insaturados e da natureza elastomérica do material.

Um pico entre 430 e 450 cm^{-1} e outro entre 580 e 610 cm^{-1} designados foram aos modos de flexão do grupo fosfato (PO_4^{3-}), especificamente correspondendo aos modos ν_2 e ν_4 comumente observados em estruturas de fosfato de cálcio. Um pico forte em 960 cm^{-1} está alinhado com o modo de estiramento simétrico do grupo fosfato (ν_1), característico das vibrações de fosfato em compostos de fosfato de cálcio. Além disso, na faixa de 1000 a 1100 cm^{-1} , foram identificados picos associados aos íons carbonato. A presença de carbonato sugere uma conversão da fase inorgânica em apatita carbonatada, em vez do β -TCF esperado, uma forma comum de fosfato de cálcio contendo grupos carbonato no lugar de alguns íons fosfato.

Nas membranas de PTFE, os espectros Raman confirmaram a estrutura do politetrafluoroetileno. Um pico próximo a 300 cm^{-1} foi associado ao estiramento esquelético da estrutura de carbono ($\nu\text{ C-C-C}$), enquanto outro, em torno de 380 cm^{-1} , correspondeu ao cisalhamento do grupo CF_2 . Além disso, um pico próximo a 571 cm^{-1} foi atribuído aos modos de flexão $\tau\text{ C-C-C}$, enquanto um pico em 730 cm^{-1} e picos proeminentes entre 1210 – 1260 cm^{-1} foram relacionados às vibrações de estiramento C–F, essencial para a estrutura molecular do PTFE.

As amostras de PTFE, especialmente quando expostas a determinados tratamentos ou fatores ambientais, podem apresentar modos relacionados a danos. Esses picos surgem devido a pequenas imperfeições ou regiões amorfas no PTFE, que podem deslocar ou criar os modos vibracionais típicos. O pico em torno de 1380 cm^{-1} pode indicar leve desordem estrutural ou estar associado à ondulação ou modos de flexão do CF_2 . Como o PTFE contém unidades repetitivas de CF_2 , vibrações ondulatórias ou de flexão podem surgir nessa faixa de número de onda.

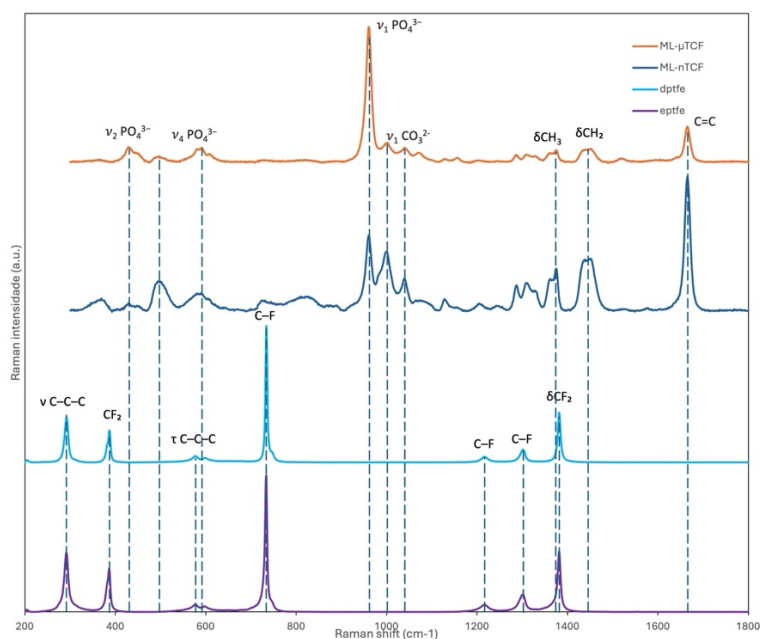


Figura 8. Espectros Raman de membranas de látex revestidas com apatita carbonatada e PTFE. Os espectros Raman confirmaram a estrutura molecular esperada e as características vibracionais das membranas de PTFE, enquanto para o ML- μ TCF e ML-nTCF os espectros indicaram que a fase inorgânica foi convertida em apatita carbonatada, como evidenciado pelos picos de carbonato na faixa de fosfato.

2.4. *As membranas experimentais demonstraram perfis distintos no suporte à proliferação de FGH-1*

Para avaliar a biocompatibilidade de membranas experimentais e comercialmente disponíveis, foram realizados ensaios de contagem de células, com resultados mostrados na Figura 9. Os ensaios de contagem de células destacaram padrões distintos de adesão e proliferação entre membranas. Em 24 horas, ML-nTCF mostrou a maior adesão celular, seguido de perto por ePTFE, indicando forte proliferação celular inicial. As membranas ML- μ TCF e dPTFE exibiram contagens moderadas de células, enquanto PS mostrou os valores mais baixos. Em 48 horas, ML-nTCF continuou a liderar na proliferação celular, com ePTFE e ML- μ TCF também mostrando crescimento significativo. dPTFE demonstrou aumentos de células mais lentos, mas consistentes, enquanto PS permaneceu o mais baixo. Em 120 horas, as membranas experimentais superaram outros grupos, confirmando seu suporte prolongado para o crescimento celular, com ML-nTCF superando significativamente PS. PS manteve sua baixa contagem de células, enquanto PTFE denso viu uma diminuição e ePTFE um ligeiro aumento.

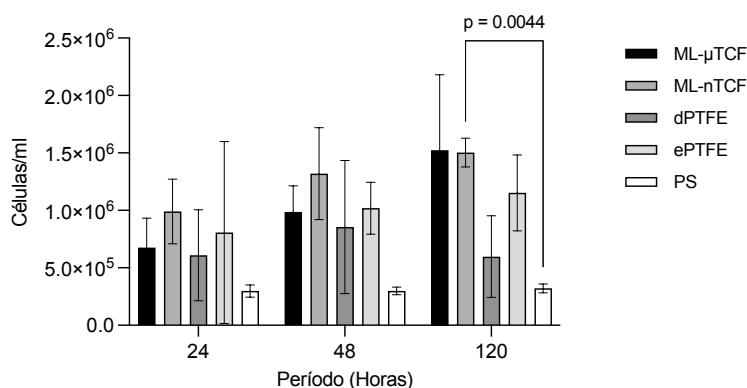


Figura 9. As membranas experimentais demonstraram perfis distintos no suporte à proliferação de fibroblastos. O gráfico de contagem de células compara a proliferação de FGH-1 nas superfícies das membranas em três períodos: 24, 48 e 120 horas. Cada cor de barra representa o número de células por mililitro de cada membrana avaliada, apresentados com valores de média e desvio padrão (DP). A análise estatística foi realizada por meio de Two-way ANOVA, e o valor de p está indicado na figura.

2.5. As membranas experimentais demonstraram perfis semelhantes ao não influenciar a citotoxicidade em FGH-1

Para obter insights sobre a citotoxicidade das membranas experimentais e comercialmente disponíveis, os níveis de lactato desidrogenase (LDH) foram medidos, e os resultados estão apresentados na Figura 10. Em 24 horas, a atividade de LDH variou entre 12-13% para as membranas experimentais e ePTFE, enquanto PS e dPTFE apresentaram níveis mais baixos, indicando citotoxicidade mínima, em torno de 7-8%. Em 48 horas, os níveis de LDH para ML- μ TCF, ML-nTCF e ePTFE diminuíram, enquanto dPTFE e PS apresentaram ligeiros aumentos, sugerindo respostas celulares semelhantes entre os materiais. Em 120 horas, os níveis de LDH para todas as membranas aumentaram ligeiramente, atingindo 11-16%. As membranas experimentais apresentaram valores comparáveis aos de PTFE e PS, apoiando sua biocompatibilidade ao longo do tempo sem diferenças significativas na resposta citotóxica entre os grupos.

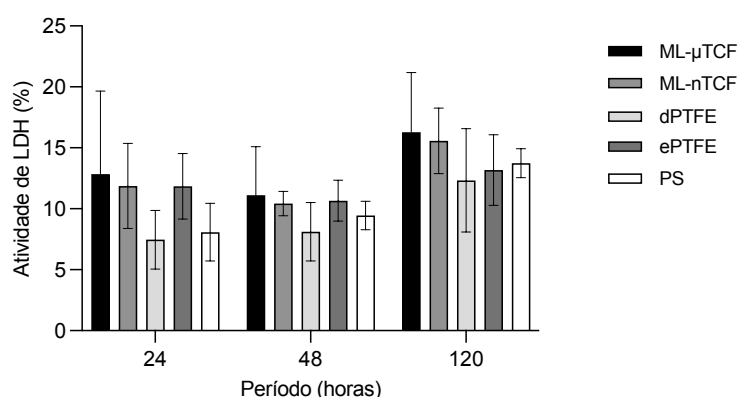


Figura 10. Os perfis de citotoxicidade das membranas foram comparáveis. O gráfico de atividade de LDH (Lactato Desidrogenase) avalia a citotoxicidade das membranas nos três períodos. As tendências e níveis semelhantes de atividade de LDH em todos os tipos de membranas, incluindo o controle, sugerem que os perfis de citotoxicidade das membranas foram comparáveis. Essa uniformidade indica que as respostas de estresse das células foram consistentes entre os diferentes materiais, refletindo a biocompatibilidade geral das membranas testadas. A atividade de LDH é apresentada em porcentagem. Cada cor de barra representa a porcentagem da atividade de LDH de cada membrana avaliada, apresentados com valores de média e desvio padrão (DP). A análise estatística foi realizada por meio de Two-way ANOVA.

2.6. Adesão celular avaliada por MEV

Um total de 10^4 células/ml foram semeadas em cada tipo de membrana e cultivadas por 24, 48 e 120 horas, com todos os experimentos conduzidos em duplicata e os resultados são mostrados nas Figuras 11 e 12. Em 24 horas, a análise de MEV revelou uma presença esparsa de células em ML- μ TCF e ML-nTCF, um número moderado em membranas de dPTFE e uma densidade celular mais alta em membranas de ePTFE em comparação com os outros tipos, particularmente as membranas experimentais. Em 48 horas, o MEV mostrou um aumento na presença de células em ML- μ TCF e ML-nTCF, com um aumento notável na adesão celular em ambas as membranas de PTFE. Em 120 horas, as células foram encontradas em todos os tipos de membrana, com a maior concentração novamente em membranas de ePTFE.

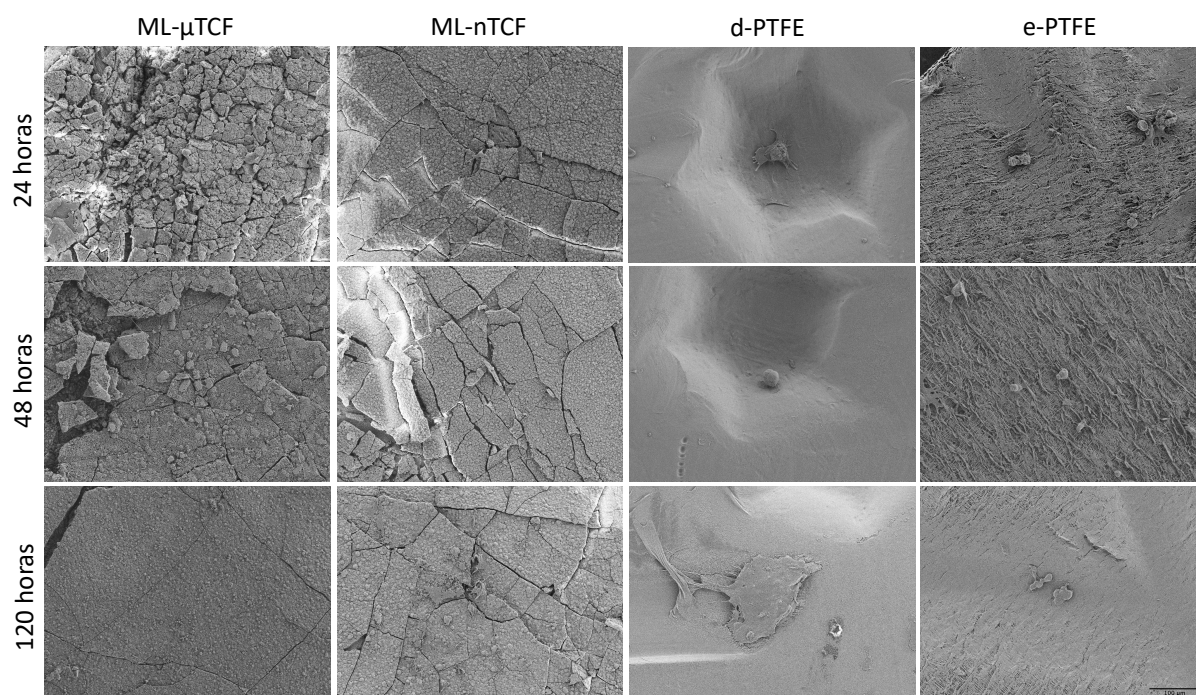


Figura 11. Imagens SEM mostrando a fixação e morfologia celular em diferentes tipos de membrana. ML- μ TCF e ML-nTCF exibem adesão celular inicial e morfologia arredondada. A membrana densa de PTFE exibe fixação celular moderada com estruturas alongadas, enquanto ePTFE mostra cobertura celular com filamentos bem definidos e adesão aprimorada, refletindo a estrutura fibrosa da superfície. A barra de escala é 100 μ m.

As células foram observadas em cada ponto de tempo, exibindo morfologias distintas dependendo do tipo de membrana Figura 12. Nas membranas de PTFE, as células exibiram filamentos alongados, aderindo bem ao material e exibindo a morfologia esperada para esse tipo de célula, especialmente em ePTFE. Em contraste, as células nas membranas experimentais eram mais arredondadas, sugerindo adesão incompleta, mas também tinham filamentos e extensões conectando-se à superfície.

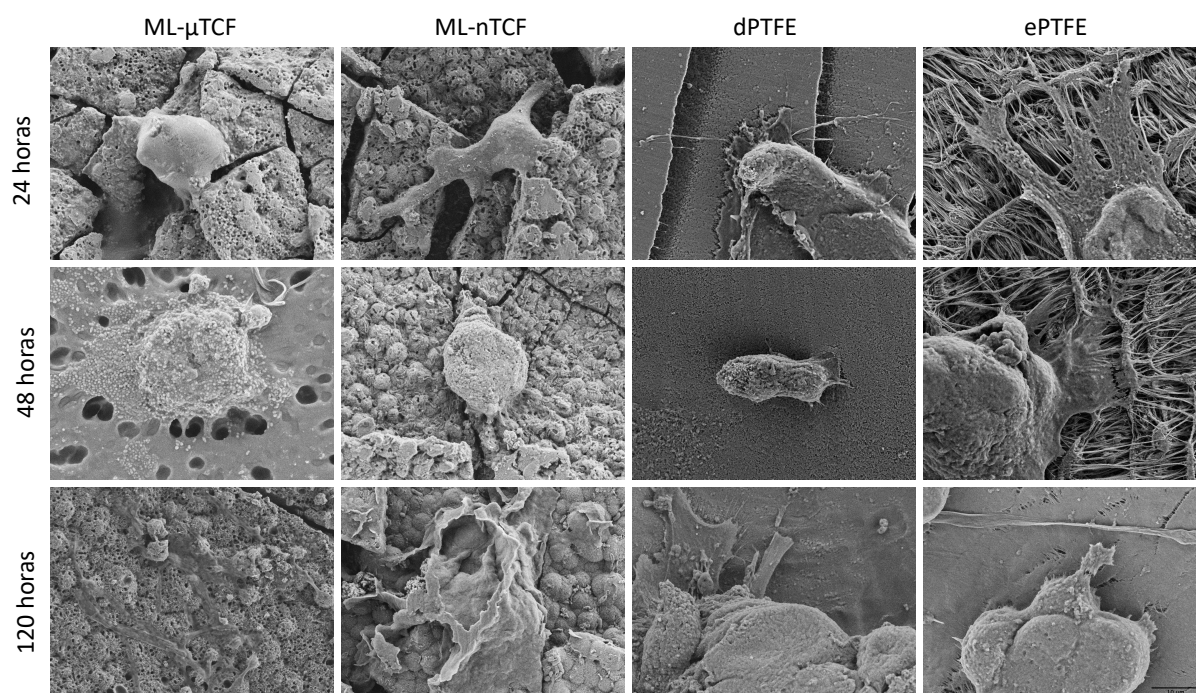


Figura 12. Imagens SEM mostrando a fixação e morfologia celular em diferentes tipos de membrana. ML- μ TCF e ML-nTCF exibem adesão celular inicial e morfologia arredondada. A membrana densa de PTFE exibe fixação celular moderada com

estruturas alongadas, enquanto ePTFE mostra cobertura celular com filamentos bem definidos e adesão aprimorada, refletindo a estrutura fibrosa da superfície. A barra de escala é 10 μm .

3. DISCUSSÃO

Este estudo avalia a eficácia das membranas experimentais ML- μ TCF e ML-nTCF em comparação às membranas tradicionais não reabsorvíveis dPTFE e ePTFE, com foco nas propriedades superficiais que influenciam o comportamento celular, especialmente para aplicações em regeneração óssea.

Uma análise por MEV revelou que as membranas ML- μ TCF e ML-nTCF apresentam alta rugosidade superficial, característica que melhora os resultados osteogênicos ao fornecer topografias em micro e nanoescala que facilitam a adesão, intransigência e diferenciação celular²⁴. Estudos indicam que fibroblastos podem alinhar-se paralelamente a microestruturas com diâmetros de 10–30 μm ^{24,25}. Superfícies rugosas revestidas com fosfato de cálcio, como as membranas ML- μ TCF e ML-nTCF, são especialmente benéficas, pois imitam a matriz extracelular, promovendo a osteocondutividade e a integração celular ao fornecer estruturas físicas para que as células ósseas se fixem e cresçam^{26–29}.

Além da rugosidade, a mobilidade das membranas experimentais destaca-se como um fator-chave^{30,31}. Como referência de ângulo de contato mostrou que as membranas ML- μ TCF eram significativamente mais hidrofílicas do que as membranas de PTFE, evidenciado por seus valores mais baixos de ângulo de contato. Superfícies hidrofílicas são conhecidas por melhorar a adesão celular inicial, promovendo a adsorção de proteínas e facilitando a fixação e a dispersão celular – etapas cruciais para a integração celular e regeneração tecidual bem-sucedida³². Esta maior hidrofilia está alinhada com estudos anteriores que demonstram influência positiva na atividade de osteoblastos e nas respostas celulares em estágios iniciais^{33,34}.

Ao comparar com membranas comerciais de PTFE, como membranas revestidas com fosfato de cálcio e com estrutura nanométrica revela vantagens específicas, particularmente no suporte à proteção e adesão celular ao longo do tempo. Uma avaliação de citotoxicidade, realizada por meio da medição da atividade de LDH, revelou variações mínimas entre os diferentes tipos de membranas, demonstrando que todas as membranas testadas, incluindo as de PTFE comerciais, apresentam biocompatibilidade comum. Esta consistência na atividade de LDH indica efeitos citotóxicos mínimos, reforçando a hipótese de que as membranas revestidas com fosfato de cálcio são alternativas viáveis aos materiais metálicos. Baixa citotoxicidade, combinada com propriedades superficiais aprimoradas, é essencial para promover a acessibilidade do material e sua integração tecidual a longo prazo^{35–39}. Esses resultados corroboram estudos anteriores que enfatizam a necessidade de biomateriais que favoreçam a terapia celular sem induzir respostas inflamatórias significativas⁴⁰.

A espectroscopia Raman confirmou os modos vibracionais característicos das membranas de PTFE, incluindo as vibrações de estiramento C–F e C–C, além dos modos de cisalhamento CF_2 e estiramento esquelético^{41,42}. Esses picos indicam uma superfície estruturalmente estável, sem

modificações inesperadas ou contaminantes, demonstrando a confiabilidade do PTFE como substrato inerte para outras aplicações de revestimento⁴³. Para as superfícies revestidas com fosfato de cálcio das membranas de látex, os espectros Raman indicaram uma composição de apatita carbonatada, com picos associados ao carbonato dentro da faixa vibracional do fosfato^{44,45}. Esse resultado pode estar relacionado ao processo de fabricação das membranas, onde o contato do β -TCF em suspensão com o meio líquido e pH neutro pode ter facilitado sua conversão em apatita carbonatada⁴⁶.

A biocompatibilidade da apatita carbonatada é atribuída à capacidade do carbonato de diminuir a cristalinidade da estrutura da apatita, aumentando sua solubilidade e favorecendo a regeneração óssea⁴⁷. Além disso, a apatita carbonatada, frequentemente encontrada em minerais ósseos naturais, é conhecida por aumentar a bioatividade e as respostas celulares devido à sua semelhança estrutural com a apatita biológica^{48–52}.

Os ensaios de previsões e contenção celular mostraram que o ML-nTCF superou as outras membranas na análise de contenção de células. Entretanto, a MEV revelou adesão celular inconsistente, com as membranas ePTFE apresentando maior número de células aderentes em todos os períodos avaliados. Essa diferença pode ser atribuída à estrutura fibrosa do ePTFE, que geralmente melhora a fixação celular^{53–58}. Apesar da hidrofília superior das membranas experimentais, os resultados sugerem que a hidrofília não é o único fator determinante no comportamento dos fibroblastos, sugerindo que a composição química pode destruir um papel mais importante^{59–62}. Fatores como densidade celular e preparo das amostras também podem ter influenciado os resultados. Estudos sugerem que a densidade celular afeta a adesão e a conformidade, destacando a importância de um controle mais específico em experimentos futuros^{63–65}. Além disso, superfícies texturizadas guiam o crescimento celular, e padrões em múltiplas escalas podem influenciar positivamente as interações celulares^{66–68}.

Os pontos fortes deste estudo incluem a caracterização detalhada das propriedades das membranas, desde a morfologia até a biocompatibilidade, fornecendo insights importantes sobre como as características da superfície afetam o comportamento celular. A comparação entre as inovadoras membranas revestidas de látex e as opções de PTFE apresenta possíveis melhorias para a engenharia tecidual. Apesar das limitações, como a natureza *in vitro* do estudo e a avaliação limitada a alguns tipos de celulares, os resultados lançam uma base sólida para pesquisas *in vivo* futuras, com foco no desempenho clínico, durabilidade e biocompatibilidade dos materiais.

As membranas revestidas com fosfato de cálcio em nanoescala, com hidrofília aprimorada, bioatividade e rugosidade estruturada, demonstram grande potencial para aplicações que requerem integração tecnológica robusta, como na odontologia regenerativa. Além disso, o uso do látex natural como base para essas membranas oferece uma alternativa econômica e promissora aos materiais tradicionais de PTFE, ampliando as possibilidades de tratamento e acessibilidade para os pacientes.

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ORIENTAÇÕES

A Pós-doutoranda Ana Flávia Piquera Santos não apresenta orientações em curso, uma vez que não está como Professora Colaboradora do Programa de Pós-graduação. Apresenta uma co-orientação em andamento de Pós-graduando do Programa de Pós-graduação em Odontologia, área de concentração em Implantodontia, orientado e tutorado pelo Supervisor.

DISCIPLINAS MINISTRADAS COM A RESPECTIVA CARGA HORÁRIA

A Pós-doutoranda Ana Flávia Piquera Santos não ministrou carga horária em Disciplinas de Pós-graduação ou graduação, bem como atividades de extensão.

COMENTÁRIOS DO SUPERVISOR

A Pós-doutoranda Ana Flávia Piquera Santos desenvolveu todas as atividades de pesquisa, ensino e extensão conforme planejado inicialmente. Apresentou nível de rendimento alto, durante execução desta pesquisa e participou ativamente das atividades do grupo de pesquisa coordenado pelo Supervisor. O artigo proveniente desta pesquisa foi concluído e está enviado a Periódico especializado com alto fator de impacto. Destaca-se a colaboração internacional do Professor Dr. Christer Dahlin da Universidade de Gotemburgo, local que a Pós-doutoranda, nos dias atuais tem realizado como Researcher pesquisa com financiamento da Osteology Foundation. Este fato comprova a capacidade e nível de excelência que a Pós-Doutoranda tem alcançado. Diante do exposto, respeitosamente recomendamos a aprovação deste Relatório Final de Pós-Doutoramento da Dra Ana Flávia Piquera Santos.

Professor Dr. Francisley Ávila Souza - Supervisor



Micro/Nano calcium phosphate coatings on latex membranes: Structural characterization and in vitro biocompatibility for guided bone regeneration

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ABSTRACT

This study investigated the biocompatibility of latex membranes (LM) coated with calcium phosphate (CaP) at the micro (LM– μ CaP) and nano (LM–nCaP) scales. Commercial dense and expanded polytetrafluoroethylene (dPTFE and ePTFE) membranes served as reference controls, while polystyrene (PS) was used as a negative control. Surface analyses confirmed the successful deposition of CaP; however, Fourier transform infrared spectroscopy (FTIR) and Raman spectroscopy revealed the formation of hydroxyapatite (HA) rather than the characteristic patterns of β -tricalcium–phosphate (β -TCP) observed in the X-ray diffraction (XRD) results prior to membrane deposition. Among the tested membranes, LM– μ CaP exhibited the greatest hydrophilicity and swelling capacity. In vitro assays with human gingival fibroblasts (HGF–1) showed that both LM– μ CaP and LM–nCaP supported cell adhesion and proliferation, with outcomes comparable to those of PTFE membranes. A statistically significant increase in cell density was observed for LM–nCaP compared to PS after 120 h. Scanning electron microscopy (SEM) revealed favorable cell–material interactions across all experimental membranes. Overall, these findings demonstrate that CaP-coated LM possess advantageous surface characteristics and cytocompatibility, highlighting their potential as effective and biocompatible alternatives to conventional PTFE membranes for guided bone regeneration.

1. Introduction

Guided Bone Regeneration (GBR) has emerged as a pivotal strategy in promoting bone gain by using membrane barriers to prevent soft tissue invasion into bone defects [1]. Multiple studies have highlighted the substantial improvement in bone formation when soft tissue encroachment is mechanically restricted. The protective function of membranes in preserving the blood clot, crucial for bone regeneration, is well-established [2–7]. Membranes not only act as barriers but also actively promote bone formation underneath, potentially through mechanisms like cell recruitment and angiogenesis [8]. Clinical

evidence corroborates the efficacy of barrier membranes in augmenting new bone formation compared to control sites, affirming their utility in bone regeneration procedures [9].

Polytetrafluoroethylene (PTFE) membranes, particularly the dense (dPTFE) and expanded (ePTFE), are widely used in GBR and guided tissue regeneration due to their non-resorbable nature, biocompatibility, and resistance to microbial infiltration [10–12]. PTFE is a highly stable polymer that remains chemically and biologically inert, effectively resisting both enzymatic and microbiological degradation [13, 14]. Fibrous structure of ePTFE allows tissue integration and cell colonization in turn improving stabilization, while smoother surface of

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PTFE reduces bacterial adhesion and the risk of infection when exposed in the oral cavity [15–17]. Both types of PTFE membranes are used in clinical settings due to their durability and predictable results in bone and periodontal regeneration procedures [18]. A notable disadvantage is its non-resorbable nature, which requires a secondary surgical procedure for removal, potentially compromising patient comfort and increasing overall treatment costs [19–21].

PTFE membranes are a gold standard in GBR procedures. However, a spectrum of synthetic and natural materials has been explored as alternative membranes in GBR procedures [22–28]. In this context, natural rubber latex (NRL) emerges as a promising candidate due to its demonstrated biocompatibility, versatile mechanical strength [29,30] and comparable performance to PTFE membranes in GBR [31]. The NRL consists in *cis*-1,4-polyisoprene extracted from *Hevea brasiliensis*, a rubber tree native to the Amazon region. It contains rubber particles, proteins, lipids, and minerals, and is collected by making incisions in the trunk [30,32]. NRL has been widely explored in biomedical applications ranging from wound healing and drug delivery to tissue regeneration and cosmetic treatments [33]. Borges et al. [34] related NRL undergoes high-speed centrifugation (22,000 $\times$ g) in the initial stages to remove the proteins (Mw $\sim$ 14 kDa) associated with contact dermatitis in order to avoid any hypersensitive process. Beyond that, sulfur is not used in NRL dressing manufacturing due to its toxicity [35]. In fact, Barros et al. [36] have applied NRL loaded with collagen to treat diabetic foot ulcer (DFU) in humans. After dressing application, we observed the skin more rigid, resilient, softer, and brighter post-wound healing, being an effective and cost-efficient alternative for DFU treatment.

An *in vivo* study using rabbit calvarial defects demonstrated that NRL significantly enhances bone regeneration and mineralization without inducing inflammation [29]. Alike, Paiva et al. [37] applied latex–collagen membrane to treat tibial bone defects in Wistar male rats, and they observed that latex's angiogenic properties and biomedical features from collagen exhibited a significant difference in the bone volume (BV)/total volume ratio (TV), maximum strength and relative stiffness analyses. On the same hand, Giovanetti et al. [38] incorporated β -Tricalcium phosphate (β -TCP) onto NRL membranes for calvarial bone defects in rats. They observed that NRL- β -TCP enhanced the bone neoformation process 56.1 % and 77.7 % in calvarial defects at 60 and 120 days, respectively. Borges et al. [39] found them biocompatible with pre-osteoblasts, showing early cell adhesion and progressive mineralized matrix formation over 21 days. The absence of cytotoxicity and support for cell growth and differentiation suggest NRL is a promising material for GBR. Furthermore, latex membranes (LM) offer a cost-effective solution, with their tunable porosity highlighting the potential of NRL to optimize drug delivery and tissue integration [29,40,41], while also supporting high cell viability, strong adhesion, enhanced proliferation, and applications in muscle tissue repair [42].

Advances in biomaterials have focused on integrating mechanical strength, bioactivity, and biodegradability to meet the complex requirements of bone tissue engineering. Titanium-ceramic composites, such as titanium-hydroxyapatite and titanium-wollastonite, have shown improved osteointegration, cellular proliferation, and surface bioactivity while retaining sufficient mechanical stability for cancellous bone applications [43]. Similarly, porous nanocomposite scaffolds incorporating bacterial cellulose, β -glucan, hydroxyapatite nanoparticles, and graphene oxide have demonstrated enhanced mechanical strength, antibacterial activity, and cytocompatibility, exhibiting optimal performance for bone regeneration [44]. More recently, a bioresorbable Mg-2Ca-1Mn-1 Sr alloy optimized via phosphorous ion implantation has exhibited refined grain structure, increased hardness, and superior corrosion resistance, underscoring its potential as a next-generation biodegradable material for orthopedic implants [45].

Recent investigations concentrated on biological effects of membranes by incorporating bioactive substances with diverse functionalities [46–49]. Calcium phosphates (CaP), particularly β -TCP, have garnered attention as potential biomaterials for bone regeneration

owing to their similarity to the bone mineral, coupled with osteoconductive and osteoinductive properties [50–55]. Its degradation occurs through cellular activity and can be modified with various ions to introduce additional properties, such as antimicrobial effects or luminescence [56]. Furthermore, β -TCP's microporous structure facilitates cell survival and neovascularization, critical for tissue regeneration [57].

The development of composite materials combining CaP with biocompatible polymers has significantly advanced GBR by improving mechanical properties, osteoconductivity, and biocompatibility [58,59]. Novel biomaterials have been studied, including bifunctional Janus membranes with osteogenic differentiation and antibacterial properties [59]; bilayer membranes incorporating nanosized CaP, increasing biomineralization [60]; and Sr-doped calcium phosphate/polycaprolactone/chitosan (Sr-CaP/PCL/CS) nanohybrid fibrous membrane that promoted osteogenic differentiation and stimulated angiogenesis [61]. In line with these advances, biopolymeric composites offer biodegradable, biocompatible, and multifunctional platforms for tissue engineering, wound healing, and regenerative medicine [62].

Furthermore, recent studies have demonstrated that hydroxyapatite (HA), the principal mineral constituent of bone and teeth, can further augment bone repair when integrated with NRL. Through a controlled, low-temperature biomimetic process in simulated body fluid, HA and related CaP phases can be deposited directly onto the NRL surface, producing a bone-like mineral layer while preserving the integrity of the underlying material. This strategy maintains the inherent flexibility and pro-angiogenic characteristics of NRL while incorporating the osteoconductive and bioactive properties of HA. Moreover, *in vitro* analyses have confirmed that these composite membranes exhibit excellent biocompatibility, with no significant hemolytic effects at clinically relevant concentrations. Collectively, these attributes underscore the potential of NRL-HA composites as promising scaffolds for GBR and related biomedical applications [63]. Accordingly, this study focuses on integrating LM with CaP at micro (μ CaP) and nanoscale (nCaP) aiming to clarify the role of coating dimensions in optimizing bone regeneration.

We examined the biological response of CaP-coated LM at both micro- (LM- μ CaP) and nanoscale (LM-nCaP), focusing on its cytotoxicity and interaction with human gingival fibroblasts (HGF-1). The adhesion and spread of cells on the membranes were assessed using Scanning electron microscopy (SEM). Finally, to visualize and analyze the structure and function of actin filaments in these cells exposed to membranes, red staining of actin was conducted alongside the arrangement of the cytoskeleton (red) and the cell nuclei (blue). Our objective is to directly apply this membrane to bone defects, facilitating a synergistic interaction between LM and CaP, to gradually release μ CaP and nCaP at the lesion site to enhance bone repair.

2. Materials and methods

2.1. Acquisition of latex and PTFE membranes

The NRL used in this study was sourced from Colitex Latex® (producer and distributor, Colina, Brazil) and derived from a blend of two clones: RRIM 600 and PB 235. To ensure stability in the liquid phase after extraction, ammonium hydroxide was added to the latex, adjusting the pH to 10.2. The latex was then centrifuged at 22,000 $\times$ g for 2 h to reduce the concentration of high molecular weight proteins, which are known to cause hypersensitivity reactions. This processing step ensured a more biocompatible material for subsequent fabrication.

Dense PTFE (Cytoplast TXT2530 -1, Osteogenics Biomedical Inc., Lubbock, TX, USA) and expanded PTFE (NeoGen™, Neoss Ltd., Harrogate, UK) membranes served as positive-control materials, selected based on their well-documented effectiveness demonstrated by previous studies [64–66].

2.2. Synthesis of Calcium Phosphate (CaP)

To prepare the CaP, calcium nitrate hydrate ($\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 99,0 %, PanReac–ITW Reagents) and phosphoric acid (H_3PO_4 , 85 % P. A., NEON, Suzano, Brazil) were separately dissolved in methyl alcohol (CH_3OH , 99.9 %, Sigma Aldrich, Saint Louis, MO, USA) under agitation and cooled in an ice bath. The H_3PO_4 solution was then slowly added to the calcium nitrate solution with continuous stirring while maintaining the ice bath, ensuring precise control over the reaction. This process was carried out to achieve Ca/P molar ratios of 1.00, 1.50, and 1.65. The resulting solution was dried at 100 °C until complete solvent evaporation occurred, producing a powder that was subsequently fractionated. The powder was then heat-treated at 1200 °C with a heating rate of 5 °C/min to obtain the β -TCP phase. Phase identity was confirmed using crystallographic data sheets from the Search Match® software database, verifying the desired composition.

2.3. LM production

The membrane fabrication process integrated both materials (Fig. 1A). First, 5 mL of NRL were deposited onto a circular plate and left to polymerize at room temperature for 24 h. After polymerization, 10 mL of an aqueous CaP suspension ($10 \text{ mg} \cdot \text{mL}^{-1}$) were introduced beneath the membrane. This setup was maintained at 50 °C for 72 h to allow the CaP to be absorbed into the latex matrix, creating a composite material with integrated properties suitable for potential biomedical applications.

Prior to all the experiments, membranes were punched to a diameter of up to 10 mm using the Acu-Punch® Skin Biopsy Punches (Acuderm® Inc., Lauderdale, FL, USA) and sterilized under ultraviolet (UV) light (YM-9007 –Guangzhou Yorkma Industrial Co. LTD., Guangdong, China) for at least 30 min each side.

2.4. Physicochemical characterization

2.4.1. SEM

For SEM analysis, samples were mounted on aluminum stubs using conductive carbon tape and coated with a 10 nm layer of gold via sputtering. SEM was performed using a Zeiss Ultra 55 FEG SEM (Leo Electron Microscopy Ltd, Cambridge, UK) in secondary electron mode, operated at 5 kV acceleration voltage and 5 mm working distance.

2.4.2. Contact Angle measurement (Wettability)

Static contact angle measurements were performed with a Biolin Scientific (Attention Theta Flow, SN TFW001594, 2023, Espoo, Finland) software using deionized water. A minimum of three membranes of each type were tested, with a single drop (5 μL) of water applied to each membrane, and the contact angle was calculated over a duration of 20 Sec, with measurements taken at intervals of 0.05 Sec. Average values are presented with standard deviation.

2.4.3. Swelling kinetics

The swelling kinetics for LM, LM- μCaP and LM-nCaP was measured by gravimetric technique, using an A&D Analytical Balance, Model HR-200 (A&D Company, Limited, Tokyo, Japan). The samples were weighed and then immersed in 2 mL of Dulbecco's Phosphate-Buffered Saline (DPBS)/modified (Cytiva-Hyclone™ Laboratories, South Logan, UT, USA) and kept at 37 °C for 24 h. The membranes were then taken off and the excess of liquid on the surface was dried twice with absorbent paper before weighing the sample. The measurements were repeated three times for each sample, and the proportion between the wet membranes and dry membranes was calculated in according to Floriano et al. [67].

2.4.4. Thermogravimetric analysis (TGA) and Derivative TG (DTG)

TGA was performed using equipment TGA 4000 (PerkinElmer, Inc.

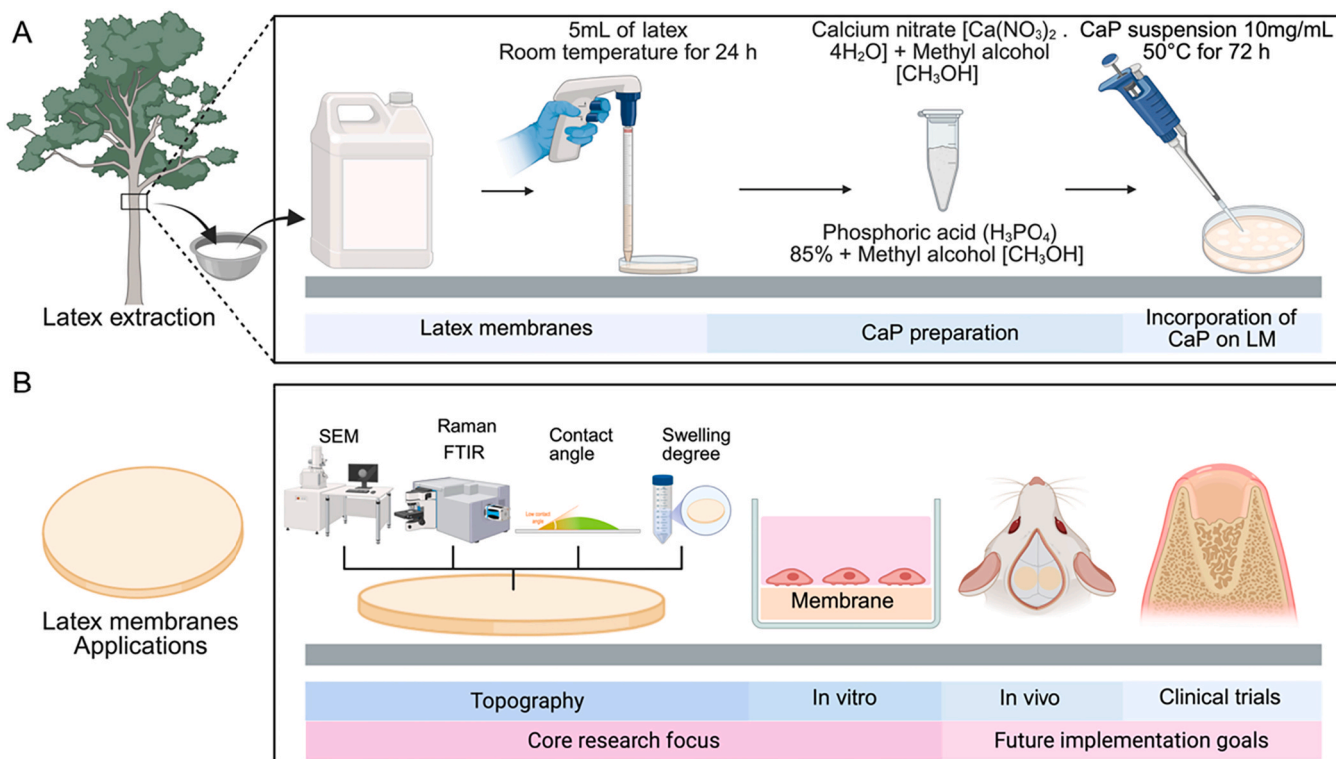


Fig. 1. Diagram of the preparation of CaP-coated LM and potential application. (A) Preparation of LM from latex extraction, incorporation of CaP and production of LM- μCaP and LM-nCaP. (B) Schematic description of the applications and future applications of LM focused on dentistry. Created with BioRender <https://BioRender.com/ign045g>.

Waltham, MA, USA), with a dry airflow of 20 mL/min. The samples were displayed in alumina sample pan (around 20 mg) and heated from 50 °C to 600 °C at 10 °C/min.

2.4.5. X-ray diffraction (XRD)

Crystalline phases were evaluated by XRD technique. XRD diffractograms were acquired using the Smartlab SE diffractometer, from Rigaku, with Cu K α radiation source. The samples were recorded in 10–60 ° of 2 θ , with a step 0.02 °. Smartlab Studio II software was used to sample indexation.

2.4.6. Fourier transform infrared (FTIR) spectroscopy

Fourier transform infrared spectra of LM, LM- μ CaP and LM-nCaP were evaluated to identify possible interactions and functional groups between the latex and the CaP. The measurements were performed in a PerkinElmer, Dual Frontier (PerkinElmer, Inc. Waltham, MA, USA), equipped with attenuated total reflectance (ATR) apparatus of zinc selenide crystal. The instrument was set to a resolution of 4 cm⁻¹ and 256 scans were recorded in the range of 4000 and 550 cm⁻¹.

2.4.7. Fourier Transform Raman Spectroscopy

For chemical composition analyze and to verify the CaP phase applied to the LM, Raman spectroscopy was performed using a confocal Raman microscope (Renishaw inVia Qontor, Renishaw plc. Wotton under Edge, UK) equipped with a 785 nm laser and Live-Track focus-tracking technology for enhanced signal stability. The laser was focused on the sample surface through a 50x/0.9 NA objective, and the Raman scattered light was collected using a Peltier-cooled charge-coupled device deep depletion near-infrared enhanced detector behind a 1200 g mm⁻¹ grating. The laser power at the sample was ~10 mW. On each sample ($n = 3$), three-point measurements were obtained at a 3 Sec integration time and 5 accumulations in each ROI. Background subtraction and cosmic ray removal for all and Raman measurements were performed in Renishaw WiRE 5.4 software.

2.5. Cell counting, viability and morphology

Primary human gingival fibroblast cells (HGF-1, passage 7, ATCC, USA) were cultured in Dulbecco's Modified Eagle Medium (DMEM 1X, Gibco, Paisley, UK) containing 10 % heat-inactivated fetal bovine serum (FBS, Gibco™—Thermo Fisher Scientific, New York, USA), and 1 % of Antibiotic—Antimycotic 100 X, containing 10,000 units/mL of penicillin, 10,000 μ g/mL of streptomycin, and 25 μ g/mL of Gibco Amphotericin B (Gibco™—Thermo Fisher Scientific, New York, USA). The cells were grown in 75 cm² culture flasks at 37 °C in a humidified incubator with 5 % CO₂. At 90–95 % confluence, cells were washed with DPBS and detached using 0.25 % trypsin—EDTA (Gibco™—Thermo Fisher Scientific, New York, USA). A total of 90 μ L of cell suspension, containing 3 $\times$ 10⁴ cells/mL, was added on the membranes. Samples were incubated for at least 2 h to allow initial cell attachment and the wells were then filled with culture medium. All experiments were performed in triplicate using a 48-well TC-treated cell culture plate (Falcon®, Corning, NY, USA) and incubated for 24, 48, and 120 h at 37 °C and 5 % CO₂. Tissue culture plate—polystyrene (PS) served as a negative control.

The number of cells for each membrane surfaces were determined using a NucleoCounter® NC-100TM system (ChemoMetec A/S, Allerød, Denmark) following the manufacturer's guidelines. After specified incubation periods, membranes were removed and placed in a clean well and 50 μ L of NucleoCounter® lysis buffer was added directly to the samples and mixing thoroughly using a vortex. Then, 50 μ L of NucleoCounter® stabilization buffer was introduced and mixing thoroughly using a vortex one more time. The prepared cell suspension was loaded into a Nucleocassette™, which had been pretreated with fluorescent propidium iodide for staining of cell nuclei. The software NucleoView was used to present the data from measurements performed

on NucleoCounter®.

Cell viability was assessed by detecting the release of lactate dehydrogenase (LDH) into the culture medium using the Lactate Dehydrogenase Activity Assay Kit (Sigma—Aldrich, Saint Louis, MO, USA), following the protocol provided by the manufacturer. Specifically, 30 μ L of supernatant from each well with the membranes were transferred to a 96-well plate (Thermo Fisher Scientific, Roskilde, Denmark) and diluted in 20 μ L of LDH Assay Buffer. Then, 50 μ L of the Master Reaction Mix was added to each well and kept protected from the light. Absorbance was measured at 450 nm every 5 min for 30 min using a FLUOstar Omega microplate reader (BMG LABTECH, Ortenberg, Germany).

2.6. Cytoskeleton Structure (F-Actin) and nuclear morphology by DAPI

To investigate cell morphology and adhesion, HGF-1 were seeded at a density of 10⁴ cells/mL in 8-well plates and cultured for 24 h until a monolayer of cells was formed. Before cell exposure, the membranes were sterilized under UV light for 60 min (on each side). The International Organization for Standardization (ISO) 10993-5:2009 method was used to perform the elution test for biomedical applications. Briefly, the proportion used was of 0.2 g of each material eluted in 1 mL of serum-free culture medium (DMEM) for 24 h at 37 °C, resulting in an eluate with a concentration of 0.2 g. mL⁻¹. Then, the eluate was used to culture HGF-1 for 24, 48 and 120 h. Brightfield images were captured using the 4 X/.13 PhL DL using a TE2000-U inverted microscope (Nikon instruments INC., Melville, NY, USA) and processed in the imaging software NIS-Elements (version 5.21.02).

Subsequently, the elutions were carefully removed, and the cells were fixed using a 4 % formaldehyde solution (HistoLab AB, Askim, Sweden) for 15 min, followed by two washes with DPBS. To visualize the adherent cells, ActinRed™ 555 ReadyProbes™ reagent (Invitrogen—Thermo Fisher Scientific, Eugene, OR, USA) was applied, selectively staining F-actin as an indicator of total adherent cells. Nuclei were counterstained with 4',6'-diamidino-2-phenylindole (DAPI) (Ibidi, Fitchburg, WI, USA). Fluorescent images were then acquired using a fluorescence microscope Eclipse Ci (Nikon Corporation, Tokyo, Japan) coupled with a digital camera DS-Fi3, using a plan fluor 20X/0.50, DIC M lens. The obtained images were processed in the imaging software NIS-Elements (version 5.30.05)

2.7. HGF-1 morphology by SEM

For a qualitative assessment of cell morphology on the membrane surfaces, SEM was performed. A total of 10⁴ cells/mL (HGF-1, passage 8) were seeded onto each membrane type and cultured for 24, 48, and 120 h, with all experiments conducted in duplicate at 37 °C and 5 % CO₂. The samples were then removed from the medium and gently rinsed with DPBS in order to detach non-adherent cells. Samples were fixed in formaldehyde 4 %, for 1 h and progressively dehydrated by sequentially immersing in an ascending series of ethanol (70, 80, 90 and 95 %), keeping them for 5 min at each concentration. Then, the samples were immersed in absolute ethanol three times, with each immersion lasting 5 min and dried at room temperature for 24 h. For SEM analysis, the membranes were mounted on aluminum stubs using conductive carbon tape and coated with a 10 nm layer of gold via sputtering using the same settings as described above (item 2.4.2).

2.8. Statistical analysis

The contact angle, swelling degree, cell counting and viability experiments were conducted in triplicate. Statistical analyses were conducted using a repeated-measures (RM) one-way ANOVA for contact angle data, and a two-way ANOVA with multiple-comparison corrections for swelling degree, cell counting, and viability. Data analyses were carried out in Prism v10 (GraphPad Software, version 10.6.0, La Jolla, CA, USA), with statistical significance defined as $p < 0.05$.

3. Results

3.1. Surface topography

The SEM of the experimental surfaces (Fig. 2A) showed the distribution of particles across the surface, creating a textured, granular landscape. Both LM- μ CaP and LM-nCaP exhibited comparable surface topography characterized by irregularly shaped pores and distinct coating layers. Successful coating was evident when compared to LM as no trace of the latex surface was visible beneath the CaP particles, confirming uniform coverage. For the dPTFE membrane, SEM showed a fibrous structure with minimal porosity; small, tightly packed pores were visible under high magnification. This dense arrangement contributes to the mechanical strength and impermeability of the membrane, making it suitable for applications requiring a robust barrier. In contrast, the ePTFE membrane exhibited a markedly different topography with an open, fibrous architecture. The expanded structure of PTFE revealed a network of interconnected pores and fibers, creating a spongy, mesh-like appearance. This structure provides high surface area and permeability, supporting fluid exchange and cellular infiltration.

Surface wettability was evaluated using contact angle measurements. Representative images of liquid droplets on the membrane surfaces are shown in Fig. 2B, while the corresponding contact angle values are presented in Fig. 2C.

The results obtained from the contact angle analysis can be directly correlated with the swelling degree results (Fig. 3A, B), highlighting the influence of surface wettability on liquid uptake behavior. Specifically, LM- μ CaP membranes exhibited the lowest contact angle values ($54.0 \pm 12.7^\circ$), indicating surface hydrophilicity, and correspondingly showed the highest swelling capacity (5 % – 7 %). Uncoated LM demonstrated a contact angle of $78.0 \pm 7.8^\circ$, along with slightly lower swelling values ranging from 5 % to 6 %. In contrast, LM-nCaP membranes, with the highest contact angle among the experimental membranes ($93.3 \pm 23.9^\circ$), exhibited the lowest swelling capacity, between 4 % and 5.3 %. The PTFE membranes, both dPTFE and ePTFE, exhibited contact angles above 90° —specifically $111.6 \pm 10.9^\circ$ and $135.5 \pm 3.2^\circ$, respectively—confirming their hydrophobic nature. Due to this

inherent hydrophobicity, swelling analysis was not performed on those membranes, as their limited liquid uptake would not yield meaningful comparative data. Statistically significant differences were observed between LM- μ CaP and both PTFE membranes and LM and ePTFE in the contact angle analysis, and swelling comparisons showed significant differences exclusively between corresponding dry and wet membranes, highlighting the distinct behaviors resulting from surface modifications.

The TGA (Fig. 3C) demonstrated comparable thermal decomposition behavior for all LM. Specifically, the LM sample displayed two distinct weight loss events at 357°C and 501°C . These events are associated with the degradation of polycis-isoprene chains and the breakdown of the highly crosslinked polymeric network, respectively. The introduction of CaP resulted in a shift of the first weight loss event to 367°C , while the second event remained unchanged at 501°C . Furthermore, the LM- μ CaP sample exhibited an additional weight loss event at 127°C , which can be attributed to the release of 6 % bound water. Finally, Fig. 3D illustrates the XRD patterns of CaP synthesized across both micro and nanoscale dimensions. The results have been properly indexed and are consistent with the CaP standard (Powder diffraction file- PDF: 9-169).

3.2. FTIR and Raman Spectra

The FTIR spectra of LM and LM-CaP membranes are shown in Fig. 4A. From the LM, the large band assigned at 3400 cm^{-1} corresponds to OH stretching. The asymmetric and symmetric stretching of C-H is observed at 2961 and 2855 cm^{-1} , respectively. The C-H₂ deformation mode corresponds to the signal at 1449 cm^{-1} . The C=C stretching is assigned to the band at 1659 cm^{-1} and the shoulder at 1716 cm^{-1} corresponds to C=O cm^{-1} . The C-C stretching at 1130 cm^{-1} and C-H out of the plane bending at 841 cm^{-1} are also observed. The LM-CaP spectra show an intense band comprising between 1032 cm^{-1} and 1040 cm^{-1} , which corresponds to P-OH stretching from the CaP phase.

Raman spectroscopy results are presented in Fig. 4B, highlighting the characteristic peaks of the NRL and CaP phases. For the LM substrate, the most prominent peak appears at $\sim 1664\text{ cm}^{-1}$, corresponding to C=C stretching (conjugated diene groups). Additional key peaks were

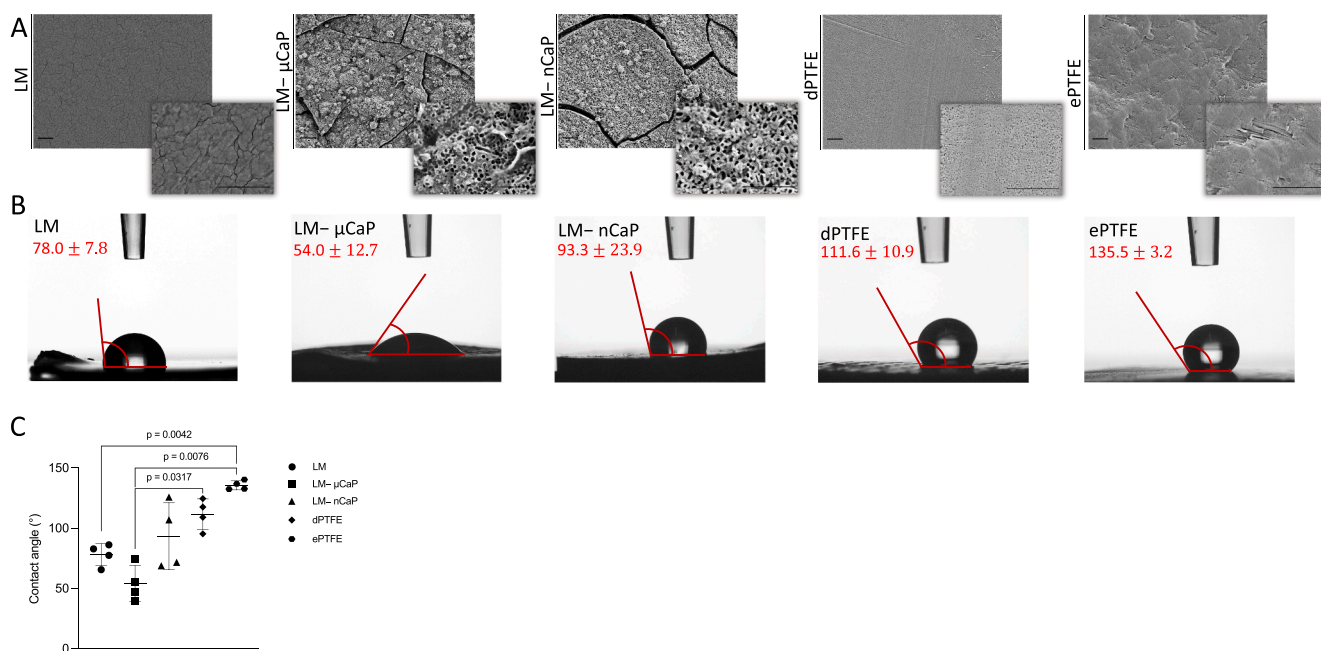


Fig. 2. Surface topography evaluated by (A) SEM; (B) representative surface contact angle image, presented with the average of contact angle values and SD; (C) contact angle values. Each format shape represents the different membranes and data are presented with mean and standard deviation (SD). Statistical analysis was carried out through a RM one-way ANOVA, and p – values are indicated. Scale bar for SEM is $5\text{ }\mu\text{m}$.

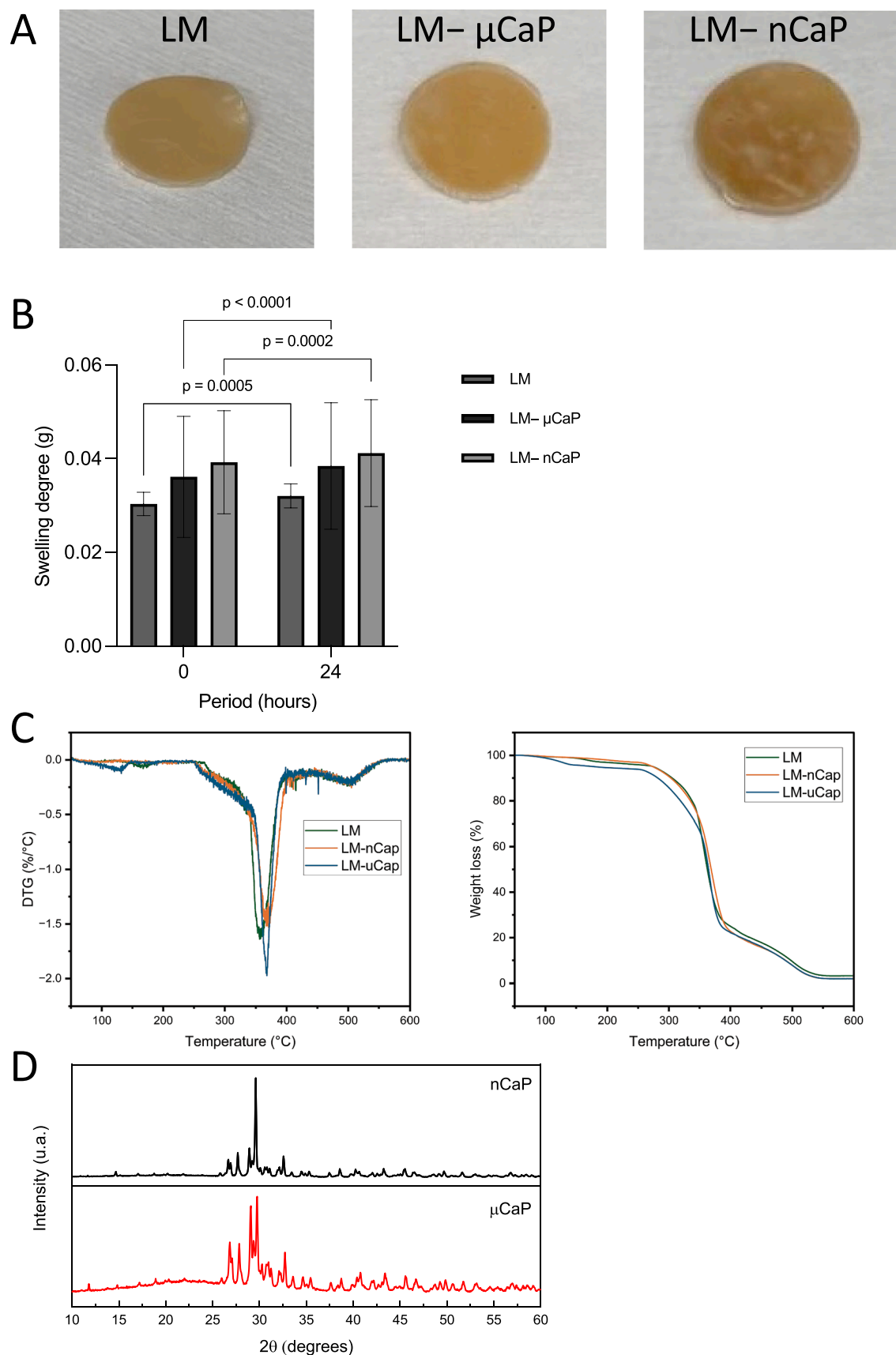


Fig. 3. Surface topography evaluated by (A) representation of the membranes after 24 h immersed in DPBS, (B) swelling degree in 0 h and 24 h; (C) TGA graphics showing no differences between the experimental membranes. Each color represents the different membranes and data are presented with mean and SD; and (D) XRD spectra of CaP samples. Each color represent the average values between three independent experiments of the different membranes and are presented with mean and SD values. Statistical analysis was carried out through a two-way ANOVA and p – values are indicated.

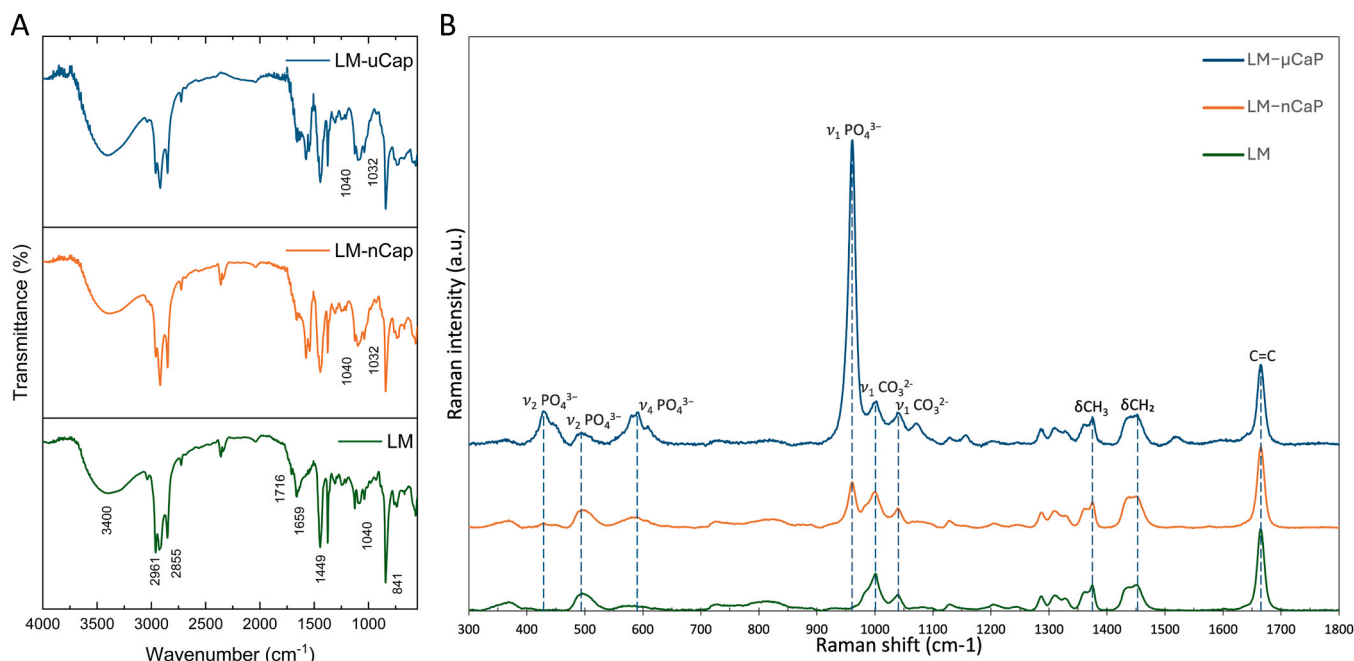


Fig. 4. FTIR (A) and Raman spectra (B) performed in order to identify the CaP phase present on the experimental membranes' surfaces.

observed at 1450, 1380, 1320, and 1000 cm⁻¹, attributed to CH₂ bending (methylene deformation), CH₃ symmetric deformation, CH₂ twisting and fanning, and C–H rocking vibrations, respectively. Notably, the peaks at 1290 cm⁻¹ and 1050 cm⁻¹ correspond to C–H bending (CH₂ wagging) in cis-1,4-polyisoprene and C–C stretching in the polyisoprene backbone, respectively. The presence of a peak at 490 cm⁻¹ suggests possible C–C skeletal deformations or interactions with inorganic fillers.

For the CaP phase in the membrane coating, the Raman spectra confirm the presence of HA rather than β-TCP. The most intense and characteristic HA peak at ~960 cm⁻¹ corresponds to the ν₁ PO₄³⁻ symmetric stretching mode, while β-TCP typically exhibits a broader ν₁ PO₄³⁻ band at ~945–950 cm⁻¹, which was not observed. Additional HA-associated peaks were identified at 430 cm⁻¹ (ν₂ PO₄³⁻ bending), 580 cm⁻¹ (ν₄ PO₄³⁻ bending), and 1040 cm⁻¹ (CO₃²⁻ substitution in B-type carbonated HA), further confirming the CaP phase composition.

3.3. Cell proliferation, cytotoxicity and morphology

The cell count assays (Fig. 5A) highlighted distinct patterns of adhesion and proliferation between membranes. At 24 h, LM-nCaP showed the highest cell adhesion, closely followed by ePTFE, indicating strong initial cell proliferation. LM-μCaP and dPTFE membranes exhibited moderate cell counts, while PS showed the lowest values. At 48 h, LM-nCaP continued to present highest values of cell proliferation, with ePTFE and LM-μCaP also showing significant growth. dPTFE demonstrated slower but consistent cell increases, while PS remained the lowest. At 120 h, the experimental membranes outperformed other membranes, confirming their prolonged support for cell growth, with LM-nCaP significantly outperforming PS. The PS maintained its consistent cell count, while dPTFE showed a decrease and ePTFE a slight increase in the cell numbers.

At 24 h, LDH activity (Fig. 5B) ranged between 12–13 % for the experimental and ePTFE membranes, while PS and dPTFE showed lower levels, indicating minimal cytotoxicity, around 6–7 %. At 48 h, LDH levels for LM-μCaP, LM-nCaP, and ePTFE decreased, while dPTFE and PS showed slight increases, suggesting similar cellular responses between the materials. At 120 h, LDH levels for all membranes increased slightly, reaching 12–16 %. The experimental membranes

demonstrated biocompatibility over time, showing comparable cytotoxicity profiles and LDH activity levels to PTFE and PS, with no significant differences in cellular stress responses across materials.

Brightfield images of the cells showed no morphological changes after 24, 48, and 120 h of culture (Fig. 5C). The morphology of HGFs remained consistent across all time points and in response to eluates from the different membranes, exhibiting a flat shape with long filaments—typical characteristics of fibroblasts. Cells were more densely concentrated in the center of the wells. On the LM-μCaP, LM-nCaP, and ePTFE surfaces, a more disorganized pattern of cell distribution was observed, whereas on dPTFE and PS surfaces, cells presented a more structured behaviour, sometimes following a circular or horizontal pattern. Notably, at 120 h on ePTFE, cells exhibited a more rounded morphology, suggesting weak adhesion or potential cell death.

Fluorescence analysis using ActinRed revealed that both LM-μCaP and LM-nCaP exhibited a well-preserved morphology, indicative of intact cytoskeletal organization (Fig. 5D). Although the overall pattern in the PS showed some differences in organization and slightly more pronounced filament extensions, the membrane compositions did not significantly alter the fundamental cytoskeletal architecture.

3.4. Cell morphology by SEM

To better understand the interaction between cells and membrane surfaces, SEM imaging was conducted, revealing key differences in cell morphology and extracellular matrix deposition (Fig. 6). In both LM-μCaP and LM-nCaP membranes, a noticeably lower number of cells was observed (white triangles). Cells on these surfaces predominantly exhibited a rounded morphology with significantly shorter filaments, often clustered near surface cracks. Furthermore, a substantial deposition of cellular matrix was observed on these surfaces, indicating the prior presence of cells, as highlighted by orange asterisks, mostly on LM-nCaP at 120 h.

In comparison, dPTFE membranes supported a relatively higher number of cells at all time points evaluated. The majority of these cells were found within the characteristic hexagonal cavities of the membrane surface, suggesting that this topography may facilitate cell entrapment. Morphologically, the cells maintained a rounded shape but extended long filaments that seems to be adhered tightly to the surface

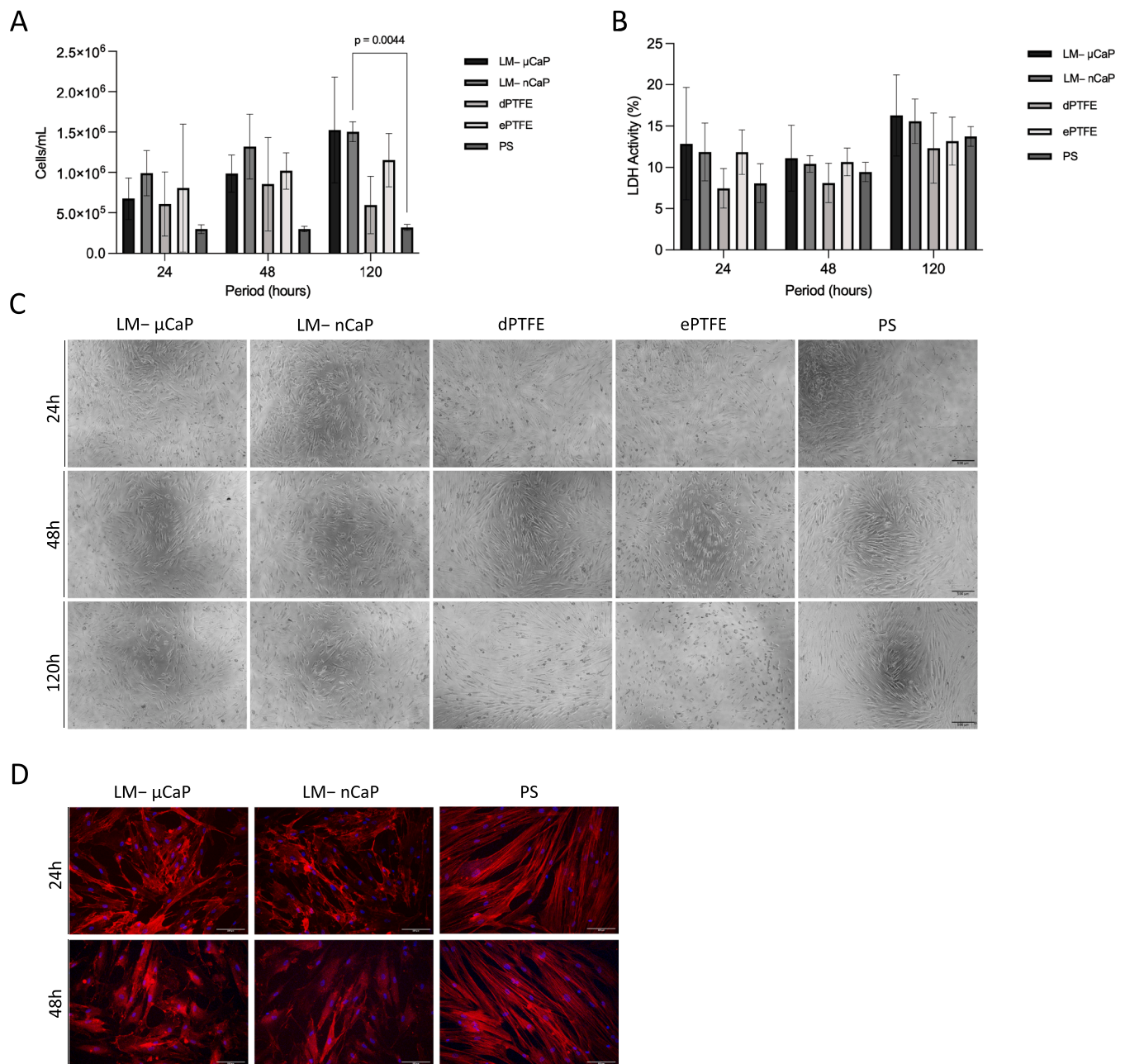


Fig. 5. The experimental membranes demonstrated distinct profiles in supporting HGF-1 proliferation and the cytotoxicity profiles of the membranes were comparable. (A) Cell counting graph compares the HGF-1 proliferation on the membrane surfaces over the evaluated periods; (B) LDH activity graph assesses the cytotoxicity of the membranes over the evaluated periods; (C) Morphology of HGF-1 when exposed to the membrane's eluates over three time points: 24, 48 and 120 h – scale bar is 500 μm; (D) ActinRed staining showing integrity in the HGF-1 morphology with the arrangement of the cytoskeleton (red) and the cell nuclei (blue), in both 24 h and 48 h – scale bar is 100 μm. LDH activity is presented as a percentage. Each color represent the average values between three independent experiments of the different membranes and are presented with mean and SD values. Statistical analysis was carried out through a two-way ANOVA and p – value is indicated.

(indicated by blue arrows), potentially increasing stability and interaction with the material. Finally, ePTFE membranes demonstrated the highest amount of cells among all surfaces tested. At the initial 24 h, the cells predominantly exhibited a rounded morphology, similar to those observed on other surfaces. However, over time, they gradually became more flattened and elongated, forming extended filaments that were tightly connected to the fibrous structures of the membrane. This progressive morphological adaptation suggests that ePTFE provides a more favorable microenvironment for cell attachment and spreading, potentially due to its fibrous architecture, which offers a greater surface area and structural support for cellular interactions.

4. Discussion

This study investigates the efficacy of the experimental LM-μCaP and LM-nCaP membranes in comparison with commercially available non-resorbable dPTFE and ePTFE membranes, with a particular focus on surface properties that modulate cellular behavior in bone regeneration applications. Specifically, surface topography, wettability, and chemical composition are examined as critical determinants of cell adhesion and interactions with biomaterial surfaces. NRL, selected as the base membrane material, provides several advantages, including elasticity and conformability for adaptation to irregular tissue surfaces, cost-

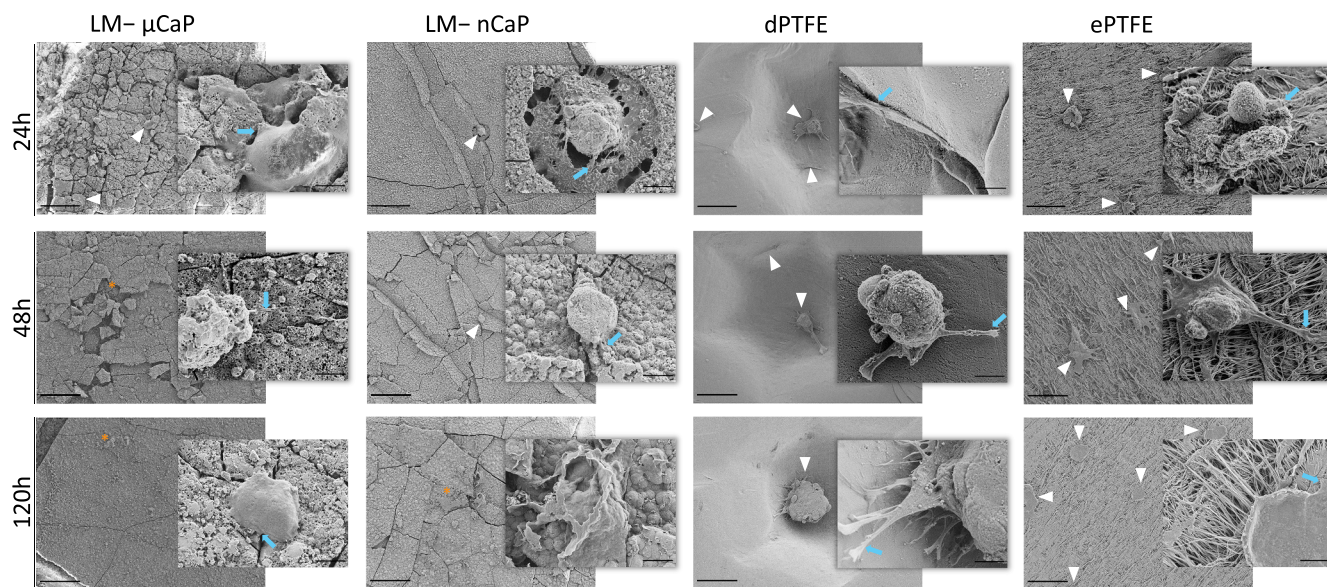


Fig. 6. SEM images showing cell attachment and morphology on different membrane types. White triangles indicate the presence of cells, while blue arrows highlight cell filaments adhered to the membrane surfaces. Orange asterisks mark cellular debris and/or extracellular matrix, suggesting prior cellular presence; however, no intact cells were observed at the site. Scale bars are 100 μm and 10 μm , respectively.

effectiveness, ease of processing, and inherent bioactivity that promotes cell adhesion and proliferation [33,39,40,42,63,68,69]. Collectively, these properties suggest that NRL-based membranes may offer superior tissue integration compared to inert materials such as PTFE, although the risk of latex hypersensitivity warrants careful consideration [70].

The ability to independently control surface chemistry and roughness—and thus tune wettability from superhydrophobic to superhydrophilic—is a powerful tool for designing biomedical surfaces that support optimal cell attachment [71]. Hydrophilic surfaces, known for their strong affinity with biological fluids, cells, and tissues, are often considered more favorable for biological integration [72,73]. However, evidence from prior studies highlights that cellular adhesion is profoundly affected by material characteristics such as chemical composition, surface topography, and internal architecture [74]. In response, surface modification methods such as plasma treatment, chemical functionalization, and bioactive coatings have emerged as effective strategies to improve scaffold bioactivity, strengthen cell adhesion, and stimulate osteogenic potential [75].

Interestingly, it was shown that fibroblast adhesion does not depend solely on wettability but follows a nonlinear pattern—hydrophilic surfaces tend to promote adhesion, but effective cell spreading is often observed on smoother, low-roughness substrates, regardless of wettability [71,76–78]. While initial fibroblast attachment benefits from optimized surface chemistry, favorable nanotopographies support long-term cell viability and proliferation [79]. Our findings demonstrated that LM surface wettability was influenced by the morphology of CaP deposition: microscale CaP (μCaP) generated more hydrophilic surfaces, while nanoscale CaP (nCaP) increased hydrophobicity (Fig. 2B, C). Comparing these results with those in Fig. 5A reveals that cell proliferation was highest on the experimental membranes, particularly LM-nCaP, when compared to the more hydrophobic PTFE surfaces.

Wettability is also closely related to the swelling behavior of polymer-based membranes, as both properties reflect the material's interaction with aqueous environments. The degree of swelling provides insight into how a membrane can absorb and retain fluids, which directly influences the release profile of loaded bioactive agents. Factors such as pH, temperature, polymer composition, and cross-linking density play significant roles in determining swelling capacity [80,81]. Excessive swelling has been associated with heightened immune responses in vivo, emphasizing the importance of controlling this property

during membrane design [82]. In this study, swelling analysis was conducted exclusively on the experimental membranes, as the hydrophobic nature of PTFE inherently limits fluid absorption. The results revealed a clear correlation between contact angle and swelling degree: more hydrophilic surfaces—LM- μCaP , LM, and LM-nCaP—exhibited higher swelling values (Figs. 2,3). However, these values remained within a moderate range across all samples.

XRD characterization was performed before CaP deposition on the membranes (Fig. 3D). The results were systematically indexed and showed consistency with the standard CaP diffraction pattern (PDF: 9–169). It is observed that as crystallite size decreases from the micrometer to the nanoscale, the diffraction peaks exhibit a tendency to broaden. Although peak broadening is the most significant effect documented, it is important to note that the relative intensities of the peaks can also change, particularly when different crystallographic planes undergo varying degrees of size reduction. A fundamental difference between the samples is evident in the relative intensity of the peaks at 29.6° and 28.9° . In the case of nanocrystalline CaP, the peak at 29.6° shows a higher relative intensity than that at 28.9° . This difference can be attributed to the increased surface area and micro-deformation characteristics associated with nanosized materials. Furthermore, variations are observed in the diffraction peaks near 29.8° , which demonstrates a broadening trend as particle size increases.

To further investigate the phase of the CaP layer on the LM, FTIR and Raman spectroscopy were employed. Raman spectroscopy confirmed that the deposited phase was carbonated HA, as indicated by the characteristic phosphate and carbonate peaks, rather than β -TCP (Fig. 4B). The unexpected formation of HA instead of β -TCP can be attributed to the greater thermodynamic stability of HA under the synthesis conditions employed—specifically, the suspension-based application, the neutral pH environment and moderate temperatures [83]. β -TCP typically forms under calcium-deficient conditions or at higher temperatures, while HA is favored when the Ca/P ratio approaches stoichiometric values. HA is the most stable and abundant phase in the inorganic bone matrix and is widely used due to its chemical similarity to native bone [55,84–86], excellent biocompatibility and osteoconductivity, though it has limited osteoinductive potential [87–89]. Advances in nanotechnology have improved HA's mechanical properties and bioactivity at the nanoscale [90,91], increasing its effectiveness in biomineralization, scaffold integration, and even enabling

applications in drug delivery [92,93] and cell targeting [94–96]. The predominance of HA in our samples therefore suggests that the material may support strong osteointegration and long-term structural stability, while potentially exhibiting slower resorption compared to β -TCP, offering a balance between bioactivity and durability for bone regeneration applications.

Incorporating HA into NRL membranes enhanced surface bioactivity. Compared to conventional PTFE membranes, LM–CaP membranes showed superior performance in supporting long-term cell proliferation and adhesion (Fig. 5A, C). In particular, LM–nCaP exhibited the highest number of cells at 24 and 48 h and remained significantly higher than PS at 120 h, indicating sustained proliferation and suggesting good biocompatibility. Cytotoxicity assessments via LDH activity showed minimal differences across membrane types, confirming that experimental membranes maintain low cytotoxicity comparable to commercial options (Fig. 5B). Low cytotoxicity coupled with improved surface properties is critical to promoting material acceptance and long-term tissue integration [97–100]. This combination of favorable surface properties and biocompatibility positions LM–CaP membranes as promising alternatives for improved tissue integration and regenerative outcomes. These findings were further supported by brightfield and fluorescence analyses (Fig. 5C, D), where the cells exposed to membrane eluates showed no visible morphological alterations.

The evaluation of cell morphology is particularly significant, as it influences cell–surface interactions. Elongated cells, characterized by cytoplasmic pseudopodia, tend to exhibit stronger attachment to titanium surfaces compared to rounded cells [101,102]. SEM analysis revealed notable differences in both cell morphology and number of adherent cells among the membranes tested. A higher number of cells with elongated morphology were observed on ePTFE membranes, whereas LM– μ CaP and LM–nCaP membranes exhibited fewer number of cells, mostly presented with a rounded morphology. These observations contrast with previous findings, which generally reported enhanced fibroblast adhesion on treated membranes compared to untreated controls, including ePTFE. For example, fibroblasts on platelet-rich plasma (PRP)-coated membranes were shown to be more embedded in the fibrous matrix and exhibited multiple thread-like extensions and anchoring points [103]. Similarly, another study reported that polylactic acid and ePTFE barriers showed minimal or no attachment of periodontal fibroblasts and osteoblast-like cells after rinsing [104].

These findings are challenging to interpret. A possible explanation for the discrepancies in the current study may be the lower cell seeding density used for SEM analysis (Fig. 6). Although intended to improve visualization of cell morphology, the reduced density may have inadvertently limited overall cell adhesion—a finding consistent with previous studies [105–107]. Furthermore, incomplete cell attachment prior to SEM preparation may have resulted in cell detachment during the rinsing steps. This effect could be attributed to inherent properties of latex—specifically serine protease—which may exhibit trypsin-like activity, potentially interfering with stable cell adhesion. It is possible that the CaP coating was insufficient to mitigate the release or activity of these substances, thereby limiting its ability to support firm cell attachment.

The strengths of this study lie in the comprehensive characterization of membrane properties, ranging from morphology to biocompatibility, providing valuable insights into how surface characteristics influence cellular behavior. By comparing the experimental membranes with established PTFE membranes, the study highlights potential advances for tissue engineering applications.

However, some limitations should be acknowledged. First, the in vitro nature of cell culture experiments may not fully replicate the in vivo environment, where numerous additional factors influence cellular responses. Nevertheless, the observed cell adhesion, proliferation, and surface apatite formation in vitro suggest good cytocompatibility and potential for controlled biodegradation, both of which are often

associated with favorable tissue integration in vivo. Translating these promising in vitro findings into clinical practice remains a challenge, as the complex in vivo scenario introduces variables such as host biological variability, dynamic mechanical forces, tissue remodeling, and immunological interactions, all of which can affect material performance. Therefore, further in vivo investigations are essential to evaluate the durability, integration, and physiological responses of these membranes under clinically relevant conditions. Second, the study focused on a limited range of cell types; examining additional cell lines would provide a more comprehensive understanding of the material's biocompatibility. Third, the mechanical properties of the membranes under physiological conditions were not evaluated, despite being critical for applications involving load-bearing implants. Finally, the antibacterial properties of the experimental membranes were not evaluated, despite their importance for implantable devices to minimize infection risks.

5. Conclusion

This study identifies LM– μ CaP and LM–nCaP membranes as promising, cost-effective, and biocompatible alternatives to conventional PTFE membranes for GBR. The results provide a solid foundation for further in vivo and clinical investigations, which will be essential to confirm their long-term performance, mechanical stability, and safety under physiological conditions, ultimately guiding their application in clinical practice.

Abbreviations

GBR	Guided Bone Regeneration
PTFE	Polytetrafluoroethylene
dPTFE	Dense polytetrafluoroethylene
ePTFE	Expanded polytetrafluoroethylene
NRL	Natural Rubber Latex
LM	Latex Membranes
CaP	Calcium Phosphate
β-TCP	β -Tricalcium Phosphate
LM–μCaP	CaP-coated LM at micro scale
LM–nCaP	CaP-coated LM at nano scale
HGF-1	Human Gingival Fibroblasts
UV	Ultraviolet
SEM	Scanning Electron Microscopy
DPBS	Dulbecco's Phosphate-Buffered Saline
TGA	Thermogravimetric Analysis
DTG	Derivative TG
XRD	X-ray diffraction
FTIR	Fourier Transform Infrared
ATR	Attenuated Total Reflection
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal Bovine Serum
PS	Polystyrene
LDH	Lactate Dehydrogenase
DAPI	4',6-diamidino-2-phenylindole
HA	Hydroxyapatite
PDF	Powder diffraction file
SD	Standard Deviation

CRediT authorship contribution statement

Christer Dahlin: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Francisley Ávila Souza:** Supervision, Methodology, Conceptualization. **Rondinelli Donizetti Herculano:** Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Rodolfo Debone Piazza:** Validation, Methodology. **Antonio Carlos Guastaldi:**

Methodology, Conceptualization. **Johansson-Loo Anna:** Methodology. **Ana Flávia Piquera Santos:** Writing – review & editing, Writing – original draft, Visualization, Software, Investigation, Formal analysis, Data curation.

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Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT 4o and 4.5 in order to improve the readability and language of the manuscript. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

Declaration of Competing Interest

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

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Data availability

Data will be made available on request.

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