POLITECNICO DI TORINO

Department of Chemical and Materials Engineering

Master of Science Course

in Materials Engineering

Master of Science Thesis

Synthesis and characterization of physically cross-linked GrapheneOxide(GO)/Collagen hydrogels for tissue engineering

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Sintesi

1. Stato dell'arte e obiettivo

L'ingegneria tissutale è una branca relativamente nuova della medicina rigenerativa sviluppatasi a partire dal 1988 con lo scopo di combinare l'uso di scaffold biocompatibili, cellule e molecole biologicamente attive per la realizzazione di modelli artificiali che possano riprodurre *in vitro* i tessuti biologici sia da un punto di vista strutturale che funzionale. E' in questo contesto che gli idrogeli hanno recentemente attratto l'attenzione della comunità scientifica.

Gli idrogeli sono reticoli polimerici idrofili in grado di cambiare le loro proprietà chimico-fisiche quando posti in ambiente acquoso. Possono infatti assorbire elevate quantità d'acqua e rigonfiarsi, dando origine ad un materiale elastico, biocompatibile e dalle proprietà meccaniche simili a quelle dei tessuti. Quando un idrogelo assorbe acqua, le prime molecole che penetrano nel network interagiscono con i gruppi fortemente polari e idrofili che lo costituiscono. In conseguenza a questa prima idratazione, l'idrogelo si rigonfia, portando ad una riduzione delle interazioni esistenti tra le sue regioni idrofobe, e consentendo pertanto l'ingresso di nuove molecole che, a loro volta, si legheranno al network. Infine, quando tutti i gruppi polari, non polari e ionici sono saturi, il restante volume libero viene occupato dalla cosiddetta *free water*.

I punti di reticolazione (*cross-linking points*) che costituiscono un idrogelo possono essere di natura chimica o fisica a seconda che le interazioni siano di tipo covalente o dovute a forze di Van der Wals, forze colombiane, legami a idrogeno e/o *entanglements*. I punti di reticolazione costituiscono un impedimento allo scorrimento reciproco delle catene polimeriche. Per questo motivo, la densità di reticolazione influenza fortemente le proprietà meccaniche dell'idrogelo e il suo rigonfiamento. All'aumentare di questo parametro, infatti, si osserva un corrispondente aumento di rigidezza del materiale nonché una ridotta capacità di rigonfiamento, essendo il network maggiormente vincolato. Inoltre, anche la natura stessa dei punti di reticolazione è determinante per le proprietà meccaniche finali. Nel caso di reticolazione chimica, l'uso di agenti reticolanti che si legano covalentemente alle catene polimeriche dà origine ad un idrogelo permanente e caratterizzato da elevate proprietà meccaniche. Ricorrendo alla reticolazione fisica, invece, la formazione di legami fisici non consente un rinforzo paragonabile al precedente e il network che ne deriva è reversibile. Allo stesso tempo, però, evitando l'uso di agenti chimici reticolanti, si esclude il comune rischio di tossicità che può limitare l'uso degli idrogeli come scaffold per la coltura di cellule e la creazione di un tessuto equivalente.

I polimeri che costituiscono un idrogelo possono invece essere naturali o sintetici. Alcuni polimeri sintetici, quali il polivinilalcol (PVA) e il polietilenglicole (PEG) per esempio, sono stati utilizzati nell'ambito dell'ingegneria tissutale grazie alla loro dimostrata biocompatibilità. Per applicazioni di questo tipo, tuttavia, è necessario soddisfare anche criteri biologici, al fine di poter promuovere l'adesione e la crescita cellulare all'interno dell'idrogelo. In questo senso, i polimeri naturali, e in particolare le biomacromolecole, rappresentano un candidato perfetto come costituenti base di bioscaffold. La loro biocompatibilità e biofunzionalità intrinseche consentono infatti di riprodurre l'interazione che biologicamente si osserva tra la matrice extracellulare e le cellule circostanti, favorendo la formazione di un tessuto equivalente.

La matrice extracellulare è la complessa microstruttura non cellulare dei tessuti che fornisce il supporto biomeccanico e biochimico alle cellule circostanti. E' costituita da diversi tipi di proteine strutturali, di cui la più abbondante è il collagene. Per questo motivo, l'utilizzo di idrogeli a base collagene permette di ricreare un ambiente tridimensionale molto simile alla matrice extracellulare osservata *in vivo,* sia da un punto di vista strutturale che da un punto di vista biofunzionale. Gli idrogeli devono infatti favorire i processi cellulari fisiologici e mantenere nel tempo lo spazio necessario a consentire lo sviluppo di un tessuto. Anche parametri fisici, quali per esempio la porosità, la densità e l'insolubilità, sono fondamentali per garantire un'omogenea migrazione cellulare e un'ampia superficie di adesione, quindi per consentire lo sviluppo di un tessuto equivalente.

Ad oggi, sono stati identificati ventotto diversi tipi di collagene derivanti dall'assemblamento di quarantasei diverse catene di collagene. Ciascun tipo viene classificato in funzione della struttura che le macromolecole finali assumono in seguito all'assemblaggio. Il collagene di tipo I, in particolare, è il più abbondante costituente dei tessuti umani ed è un cosiddetto *fibril forming collagen*. Esso deriva infatti dall'organizzazione di tre sequenze amminoacidiche sinistrorse in un'unica tripla elica destrorsa, all'interno della quale le catene interagiscono e si consolidano tramite legami a idrogeno. Una macromolecola di collagene misura tipicamente 300 nm in lunghezza e 2nm in diametro. Le triple eliche così formate si auto-assemblano secondo un processo definito di *fibrillogenesi* portando alla formazione di fibrille, che a loro volta si organizzano ulteriormente in fibre. Le fibrille presentano diametri variabili tra i 10 e i 300 nm e una lunghezza di centinaia di micron, mentre le fibre hanno diametri dell'ordine dei micrometri e lunghezze dell'ordine dei millimetri o centimetri.

Nel caso di collagene estratto dai tessuti biologici per uso sperimentale, il processo di fibrillogenesi viene indotto *in vitro* a 37°C in seguito alla neutralizzazione della soluzione acida in cui la proteina viene stoccata per la conservazione. Gli idrogeli che ne derivano, tuttavia, presentano proprietà meccaniche nettamente inferiori rispetto a quelle dei tessuti biologici di origine, a causa del processo di estrazione a cui il collagene viene sottoposto. A differenza delle triple eliche, infatti, i legami a idrogeno e i legami covalenti nativi rotti durante il processo non vengono ripristinati durante il processo di fibrillogenesi ricreato *in vitro*, portando alla formazione di un materiale dalle proprietà notevolmente ridotte.

Le scarse proprietà meccaniche rappresentano il principale limite legato all'uso degli idrogeli di collagene come scaffold per l'ingegneria tissutale. Le cellule, introdotte all'interno di un idrogelo di collagene prima che la fibrillogenesi avvenga, interagiscono infatti con il network tridimensionale, che ne determina l'adesione, la migrazione e la crescita. Quest'ultima, in particolare, induce dei processi di rimodellamento e compattazione dell'idrogelo dovuti alle forze di trazione esercitate dalle cellule in crescita. La contrazione si verifica nel momento in cui queste forze di trazione superano la resistenza del network e la velocità con cui avviene nel tempo dipende dalla concentrazioni di collagene e di cellule iniziali. In particolare, la maggior contrazione si osserva in caso di alto contenuto di cellule e basso contenuto di collagene, viceversa per ottenere la minor contrazione. Le proprietà meccaniche del network influenzano dunque tutti i processi cellulari che portano alla formazione del tessuto equivalente finale. È stato infatti dimostrato che la rigidezza della matrice extracellulare influenza fortemente i processi cellulari che si instaurano a contatto con essa. All'aumentare della rigidezza vengono favorite l'adesione, la crescita, la migrazione e la differenziazione cellulare. È stato per esempio osservato che i tessuti tumorali presentano una rigidezza circa 10-20 volte superiore rispetto a quella dei tessuti normali dovuta alla maggior concentrazione di collagene presente, il che giustifica la velocità con cui notoriamente i tessuti tumorali si sviluppano e crescno. Per questo motivo, la possibilità di controllare e migliorare le proprietà meccaniche degli idrogeli di collagene è diventata di primaria importanza in questo campo di ricerca.

Come precedentemente anticipato, il rinforzo può avvenire sia tramite cross-linking chimico che attraverso cross-linking fisico. A differenza degli idrogeli derivati da polimeri artificiali, quelli a base collagene non richiedono reticolazione per poter essere formati, ma solamente per essere rinforzati. Essendo la reticolazione chimica strettamente legata all'uso di agenti reticolanti, il ricorso ad un rinforzo favorito dall'instaurazione di interazioni fisiche è da preferirsi nel caso di idrogeli di collagene, considerata la loro ampia applicazione come scaffold per la coltura cellulare.

Negli ultimi anni, il grafene e i suoi derivati hanno suscitato particolare interesse nel campo della medicina rigenerativa, grazie alle loro peculiari proprietà quali elevata estensione superficiale, elevato fattore di forma, eccellenti proprietà meccaniche, elettriche e ottiche. A queste si aggiungono elevata purezza, buona bio-funzionalizzabilità, elevata capacità di carico di farmaco e facilità nel penetrare le membrane cellulari, che rendono questi nanomateriali biocompatibili e adatti al rilascio di farmaco, alla biosensoristica e al controllo della crescita cellulare. Tra i vari derivati del grafene, l'ossido di grafene presenta anche una buona disperdibilità e stabilità grazie alla presenza di gruppi carbonili, idrossili ed epossidici sulla sua superficie. Questi gruppi contenenti ossigeno agiscono anche da siti di ancoraggio per la l'adesione e crescita cellulare, nonché per l'instaurazione di legami fisici o chimici tra GO e le proteine, quali per esempio il collagene. La capacità dell'ossido di grafene di stimolare le attività cellulari è una prova della sua biocompatibilità, ma particolare attenzione va prestata anche allo studio della citotossicità, che è stata dimostrata essere dipendente dalla sua concentrazione, taglia e numero di layer e dal tempo di permanenza nell'ambiente di coltura. Questo materiale è stato inoltre riconosciuto come efficace rinforzante per bioscaffold.

Il presente lavoro di ricerca si incentra sulla sintesi e caratterizzazione di idrogeli nanocompositi di collagene caricati con ossido di grafene a concentrazione crescente, allo scopo di migliorarne la rigidezza. Tali idrogeli sono stati ottenuti sfruttando il processo di fibrillogenesi del collagene a 37°C e utilizzando l'ossido di grafene come rinforzante e reticolante fisico tra le fibre di collagene, evitando così l'uso di reticolanti chimici che avrebbero potuto comprometterne l'applicazione come scaffold per la coltura di cellule. Dopo un breve elenco dei materiali utilizzati per questo studio, vengono illustrati i metodi sperimentali e i corrispondenti risultati con annessa discussione, prima di trarre le conclusioni finali. Le proprietà meccaniche e morfologiche e il possibile utilizzo in ambiente cellulare per la sintesi di tessuti equivalenti sono state valutate.

2. Materiali

Nitta Gelatin Inc.® Collagene di tipo I-A 3mg/ml, *NiSiNa Materials* Ossido di Grafene (GO), *Nitta Gelatin Inc.*® buffer, mezzo di coltura di Dulbecco modificato (DMEM) 5x, EtOH 85%, HCl 10^{-3}M, *Sterlab Scientific*® filtri sterili in PES con porosità di 0.22μm, linea cellulare NIH3T3, *Corning*™ piastre per coltura cellulare con 24 pozzetti trattate e non trattate.

3. Metodi sperimentali e risultati

3.1. Sterilizzazione della sospensione di GO

L'applicazione degli idrogeli di collagene e ossido di grafene come scaffold per la coltura cellulare richiede che tutti i componenti con cui vengono realizzati siano sterili. Per questo motivo, la sterilizzazione della sospensione di ossido è uno step fondamentale per la riuscita delle analisi sperimentali.

La sterilizzazione è stata condotta attraverso successive sospensioni in etanolo (EtOH) concentrato all'85%. EtOH 70% viene solitamente utilizzato per mantenere sterile la *clean bench* in cui vengono manipolate le cellule in coltura. L'etanolo e il mezzo di coltura (DMEM) sono stati preventivamente sterilizzati attraverso filtri con pori del diametro di 0.22 μm, che non potevano invece essere utilizzati per l'ossido essendo questo di taglia maggiore. La sospensione originale è stata dapprima centrifugata per rimuovere l'acqua presente come mezzo di sospensione. Dopodiché, l'ossido è stato sospeso in EtOH 85% e sottoposto a numerosi risciacqui tramite l'uso di una pipetta, prima di essere nuovamente centrifugato per separarlo dall'alcol. Questa operazione è stata ripetuta tre volte per garantire la totale sterilizzazione. Successivamente, l'ossido è stato risciacquato con DMEM per rimuovere ogni traccia di etanolo. Anche questa operazione è stata ripetuta tre volte, alternata a centrifugazioni, per garantire un opportuno lavaggio ed evitare la presenza di agenti chimici tossici residui.

Ciascun passaggio è stato condotto all'interno della camera pulita per evitare contaminazioni. Le centrifugazioni sono state condotte a 14000 rpm e 4°C per 30 minuti. La sospensione finale in DMEM è stata operata in modo da riportare la sospensione allo stesso volume, e dunque alla stessa concentrazione, di partenza. A causa dell'accidentale rimozione ad ogni passaggio di piccoli quantitativi di ossido non ben sedimentati, la concentrazione finale dell'ossido in sospensione era visibilmente inferiore rispetto a quella inziale. Per questo motivo, la sospensione è stata analizzata tramite termogravimetria in modo da valutare con esattezza il contenuto di GO per un dato volume di sospensione, e quindi correggerne la concentrazione.

L'efficacia di questo metodo di sterilizzazione è stata successivamente testata tramite l'uso degli idrogeli caricati con GO come substrati per la coltura cellulare.

3.2. Sintesi degli idrogeli nanocompositi di collagene e GO

Gli idrogeli sono stati preparati seguendo il protocollo fornito da *Nitta Gelatin Inc*®, che prevede il mescolamento di collagene 3 mg/ml di tipo I (A) con il mezzo di coltura di Dulbecco modificato (B), seguito poi dall'aggiunta del buffer (C) secondo il rapporto A:B:C=7:2:1. Il processo è stato condotto tenendo le soluzioni su ghiaccio in modo da evitare che la gelazione

avvenisse prima che tutti i componenti fossero stati opportunamente mescolati. Gli idrogeli di collagene così ottenuti sono stati usati come riferimento per valutare le differenze rispetto a quelli caricato con ossido di grafene. In questo caso, alcune modifiche sono state apportate al suddetto protocollo per aggiungere l'ossido senza intaccare il rapporto 7:2:1, così da garantire la stessa concentrazione finale di collagene nell'idrogelo. Sempre tenendo le soluzioni in ghiaccio, sono stati dapprima mescolati l'ossido di grafene (preventivamente sospeso in DMEM 5x anziché in acqua) e il DMEM 5x. In particolare, la quantità di GO necessaria ad avere una sua determinata concentrazione nell'idrogelo è stata sottratta al volume di DMEM 5x previsto, in modo che nel complesso i due componenti dessero un volume finale tale da mantenere il rapporto 7:2:1. A questo punto, è stato aggiunto il collagene e, dopo mescolamento fino all'omogeneità, il buffer, prima di portare in forno a 37éC per 30 minuti.

Sono stati preparati gel del diametro di 8 mm e con concentrazione di GO crescente e compresa tra 0.05 e 5% w/w. La concentrazione in peso di ossido di grafene è stata valutata rispetto al quantitativo di collagene utilizzato, essendo lo scopo di questo studio quello di valutare l'effetto di GO su collagene e sugli idrogeli che ne derivano. Il protocollo modificato si è dimostrato efficace come quello originario.

3.3. Swelling degli idrogeli nanocompositi

Il quantitativo di acqua assorbito da un idrogelo (*swelling*) è un indicatore qualitativo della densità di cross-linking dell'idrogelo stesso. All'aumentare di questa, infatti, diminuisce la quantità di acqua che può essere assorbita dal gel, essendo i punti di reticolazione un vincolo allo scorrimento reciproco delle catene e quindi allo swelling.

Idrogeli con una concentrazione di GO pari a 0.05, 0.1, 0.2, 0.5, 1, 2 e 5% w/w sono stati preparati, oltre ad un idrogelo di collagene di riferimento. In particolare, tre campioni per ciascun tipo sono stati prodotti. Tutti i campioni sono stati pesati al termine dei 30 minuti richiesti per la gelificazione, e di nuovo dopo essere stati essiccati a temperatura ambiente e in vuoto per una notte. Si indicano rispettivamente w_w e w_d il peso dei gel ancora idratati e di quelli essiccati, w_0 invece il peso del contenitore in cui sono stati inseriti. La percentuale di acqua assorbita dai gel viene pertanto definita secondo l'equazione 3.1:

$$\%water = \frac{Ww-Wd}{Ww-W0}\text{x}100 \qquad (3.1)$$

Una valutazione delle percentuali di acqua dei suddetti idrogeli ha portato ai risultati mostrati in figura 3.1. Nonostante non sia chiaramente visibile a causa dell'ampia scala utilizzata rispetto alle piccole variazioni riscontrate, l'ossido di grafene ha portato ad una generale seppur contenuta riduzione del contenuto di acqua rispetto a quello dell'idrogelo di collagene di riferimento. Secondo quanto riportato in precedenza, questa riduzione corrisponde ad un aumento della densità di cross-linking, che in questo caso è legato alla presenza dell'ossido che agisce da rinforzante e reticolante fisico. Un picco di riduzione è stato in particolare osservato in corrispondenza di una concentrazione di GO pari allo 0.5%, ossia una concentrazione intermedia. Ciò potrebbe risultare controintuitivo, dal momento che l'aggiunta di un filler porta solitamente ad un aumento della densità di reticolazione all'aumentare della sua concentrazione. È importante però ricordare che la matrice in questo caso è costituita da collagene, proteina che è soggetta ad un naturale processo di auto-assemblamento in fibrille e

fibre durante la formazione dell'idrogelo. Si presuppone, pertanto, che l'andamento dello *swelling* con la concentrazione di GO sia strettamente legato all'effetto che l'ossido ha sul processo di fibrillogenesi del collagene.

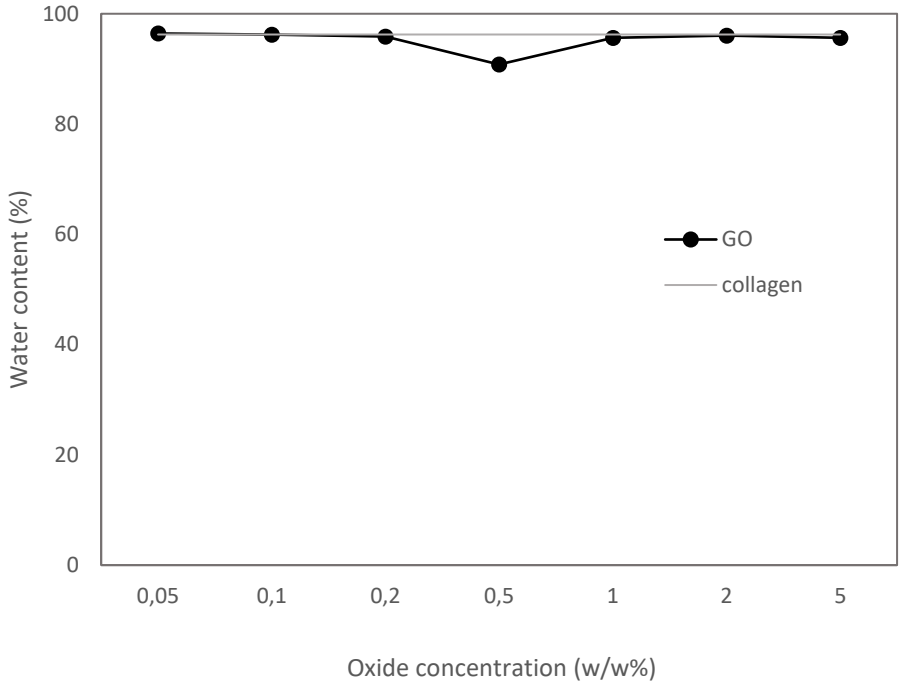


Fig. 3.1 Contenuto di acqua di idrogeli di collagene caricati con ossido di grafene a concentrazione crescente compresa tra lo 0.05 e il 5%. L'andamento è posto a confronto con la percentuale osservata nel caso di un idrogelo di collagene di riferimento, pari al 96%.

L'ipotesi avanzata è che l'ossido di grafene dia origine a due effetti opposti quando usato come rinforzante in un idrogelo di collagene: un effetto che prevale a basse concentrazioni per cui l'ossido agisce da reticolante fisico aumentando l'interazione interfibrillare, e un effetto che prevale ad alte concentrazioni per cui l'elevato contenuto di ossido agisce da impedimento per il processo di fibrillogenesi del collagene. Questo spiega perché, per concentrazioni di GO superiori allo 0.5%, si osserva un ritorno a percentuali di acqua assorbita dagli idrogeli simili a quella del campione di riferimento.

3.4. Dicroismo circolare

Il dicroismo circolare è un'analisi spettroscopica ampiamente utilizzata per lo studio della struttura secondaria delle proteine e dei polipeptidi in soluzione. Essa permette di distinguere strutture ordinate del tipo *α-helix* e *β-sheet* da quelle disordinate di tipo *random coil.* L'analisi consiste nel valutare la differenza di assorbimento a diverse lunghezze d'onda di un fascio di luce polarizzata circolarmente a sinistra (L-CPL) e di una polarizzata circolarmente a destra (R-CPL) da parte di molecole otticamente attive come il collagene. Le proteine contengono infatti uno o più gruppi cromofori. I gruppi cromofori amminici delle proteine, in particolare, dominano gli spettri di dicroismo circolare a lunghezze d'onda inferiori ai 250 nm.

La struttura secondaria è sensibile all'ambiente circostante, in particolare a temperatura, pH e forza ionica della soluzione. Nel nostro caso l'effetto studiato è quello legato alla presenza di ossido di grafene nella soluzione di collagene, ad una temperatura di 4°C e pH neutro.

Le misurazioni sono state condotte su una soluzione di collagene di riferimento e poi sulla stessa soluzione in presenza di GO a concentrazioni pari allo 0.5, 2 e 5 % [fig. 3.1] per valutarne l'effetto sull'intensità e posizione dei picchi che caratterizzano la struttura del collagene. Il collagene presenta infatti due picchi tipici, uno positivo a circa 222 nm relativo alla presenza di triple eliche e quindi rappresentativo dell'attività biologica della proteina, e uno negativo intorno ai 198 nm legato alla presenza di poliprolina. È stato riportato in letteratura che, in caso di completa denaturazione del collagene, il picco positivo scompare mentre quello negativo subisce una traslazione verso lunghezze d'onda maggiori.

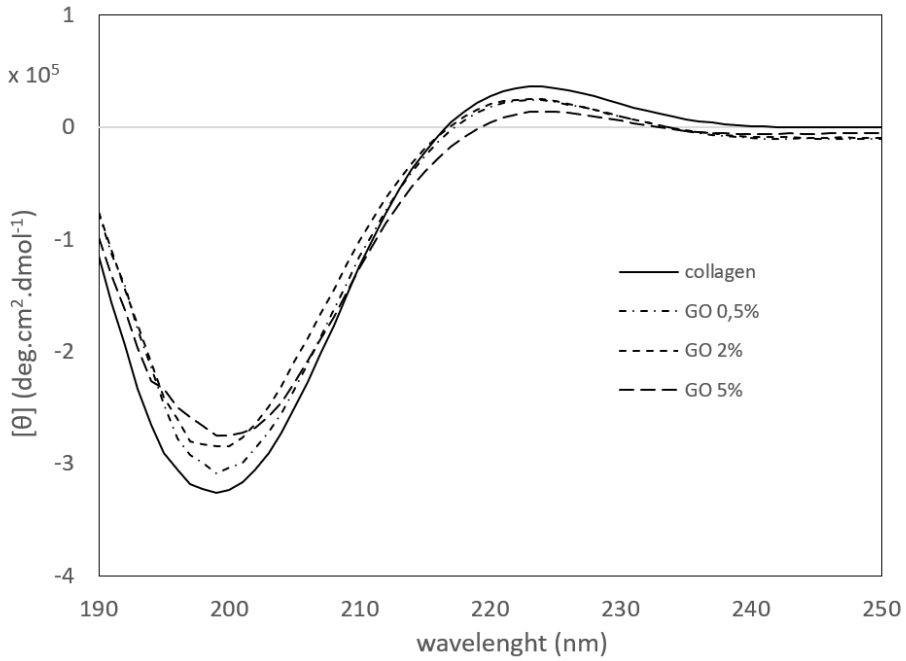


Fig. 3.2 Spettroscopia di dicroismo circolare di una soluzione di collagene 0.2 mg/ml e della stessa soluzione in aggiunta di GO 0.5%, 2% e 5% w/w.

Per tutti i campioni analizzati è possibile individuare la presenza del picco a 222 nm, a conferma del fatto che nessuna delle concentrazioni di GO utilizzate ha causato una completa denaturazione del collagene e che quindi le sue funzioni biologiche sono state preservate anche in presenza dell'ossido, requisito fondamentale per le applicazioni previste. Nonostante ciò, il picco mostra un'intensità decrescente all'aumentare della concentrazione di ossido, che quando presente al 5% comporta una lieve traslazione del picco negativo verso destra seppur in modo non significativo. In generale, questi due fattori permettono di intuire che la percentuale di triple eliche presenti in soluzione diminuisce all'aumentare della concentrazione di ossido di grafene, e non perché la concentrazione di proteina sia diminuita. L'ellitticità è stata infatti valutata come ellitticità molare [θ], ossia normalizzata rispetto alla concentrazione di collagene e quindi indipendente da questa. In tal modo, è stato possibile valutare l'effetto della quantità di ossido presente senza tener conto della variazione del contenuto di collagene che ne è derivata. Per determinare l'effettivo contenuto di triple eliche è stato calcolato l'Rpn di ciascun campione, ossia il rapporto tra l'ellitticità in corrispondenza del picco positivo a 222 nm e di quello negativo a 198 nm. L'Rpn per il campione di riferimento contenente solo collagene è pari a

0.11. In seguito all'aggiunta di GO 0.5, 2 e 5%, il valore è sceso rispettivamente a 0.108, 0.105 e 0.103. Normalizzando questi ultimi rispetto al valore relativo al riferimento, si è potuto osservare che le concentrazioni crescenti di ossido hanno ridotto il contenuto di triple eliche rispettivamente al 98%, 95% e 93% del valore iniziale.

Si può pertanto concludere che la struttura secondaria del collagene è stata modificata seppur in modo non significativo, e che l'aumento del contenuto di acqua degli idrogeli per concentrazioni di GO superiori allo 0.5% è dovuta al fatto che la fibrillogenesi del collagene è progressivamente ostacolata dall'aumento del GO inserito, a conferma di quanto precedentemente ipotizzato.

3.5. Reologia

La valutazione delle proprietà reologiche degli idrogeli è fondamentale per simulare le condizioni a cui questi vengono sottoposti durante la loro compattazione, indotta dagli sforzi di trazione esercitati dalle cellule in crescita. Queste possono infatti esercitare deformazioni fino ad un valore di 0.5 a frequenze comprese tra 0.1 e 10 Hz, ossia circa tra 0.6 e 60 rad/s.

Il comportamento reologico degli idrogeli è stato valutato a 25°C con un reometro a piatti piani paralleli. Il modulo di immagazzinamento (*storage modulus*) e il modulo di perdita (*loss modulus*) sono stati studiati in un range di frequenza angolare compreso tra 1 e 100 rad/s e una deformazione costante del 5% (*strain controlled analysis*) nel campo di viscoelasticità lineare preventivamente valutato. Il gap tra i piatti del reometro è stato fissato ad 1 mm. Sono stati testati un idrogelo di collagene di riferimento e idrogeli a concentrazioni crescenti di GO pari a 0.5, 2 e 5% [fig. 3.3].

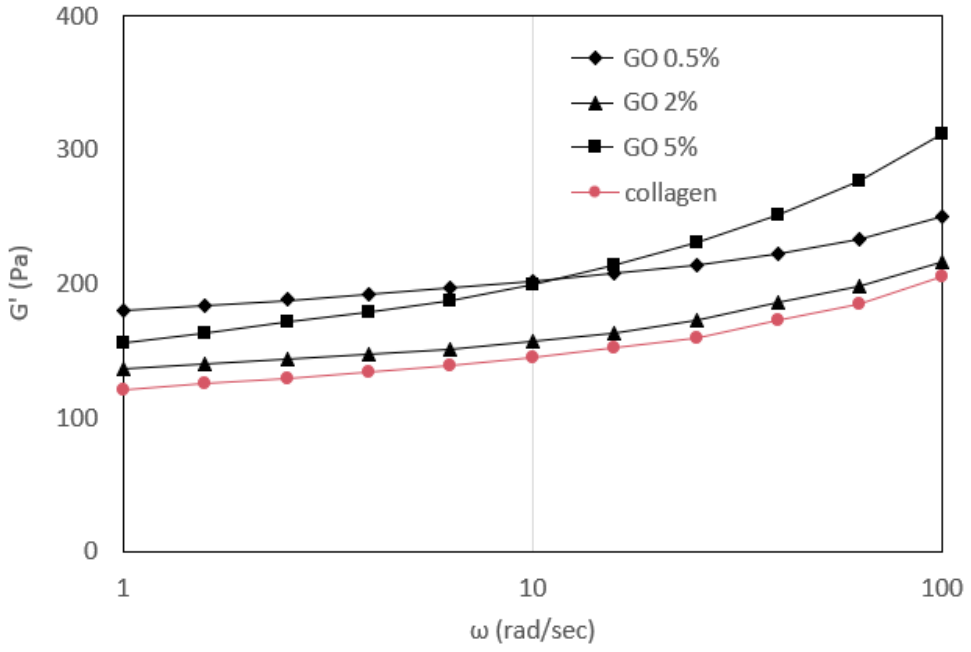


Fig. 3.3 Risposta viscoelastica di idrogeli caricati con concentrazioni crescenti di GO rispetto ad un idrogelo di collagene di riferimento. La dipendenza del modulo di immagazzinamento G' dalla frequenza è stata testata ad un valore costante di deformazione pari al 5%.

Come si può osservare dal grafico sopra riportato, G' dipende dalla concentrazione di GO all'interno dell'idrogelo. In particolare, una concentrazione di GO pari allo 0.5% ha portato ad un aumento di G' pari al 50% (180Pa) rispetto all'idrogelo di collagene di riferimento (120 Pa). GO al 2% invece ha provocato un aumento del solo 12,5 % (135 Pa). Ci si sarebbe dunque aspettati un'ulteriore diminuzione del valore di G' nel caso di una concentrazione pari al 5%. Al contrario, un aumento del 29% (155 Pa) è stato riscontrato. Questo comportamento è presumibilmente dovuto a due effetti che GO può avere sugli idrogeli: se da un parte l'elevato contenuto di GO inibisce la fibrillogenesi del collagene come precedentemente ipotizzato, dall'altra invece è responsabile di un aumento della rigidezza del materiale dovuta all'elevata resistenza meccanica intrinseca che l'ossido di grafene possiede e che diventa sempre più prevalente man mano che esso aumenta in concentrazione. Fatta eccezione per una concentrazione del 5%, per il motivo appena illustrato, l'andamento decrescente di G' all'aumentare della concentrazione dell'ossido è un'ulteriore supporto alle ipotesi precedentemente avanzate tramite lo studio del contenuto d'acqua degli idrogeli e la valutazione degli spettri di dicroismo circolare.

3.6. SEM

Immagini al microscopio elettronico a scansione (SEM) sono state catturate per studiare la morfologia interna degli idrogeli compositi. In questo caso, solo concentrazioni di GO pari allo 0.5 e al 5% sono state utilizzate, essendo una tecnica che permette di valutare solo visivamente e qualitativamente la densità di reticolazione. I campioni sono stati disidratati attraverso successive immersioni di un'ora in etanolo a concentrazione crescente dal 60 al 100% a 4°C, dopo aver preventivamente fissato la struttura interna attraverso l'uso di glutaraldeide. Dopodiché, i gel sono stati liofilizzati ad una pressione di 10 Pa e una temperatura di -80°C per rimuovere definitivamente la presenza di acqua, tagliati, fissati sul portacampioni con un adesivo al carbonio e rivestiti con oro tramite sputter per poter essere osservati.

Dalle immagini [fig. 3.4] è possibile identificare le fibre di collagene e la struttura porosa interconnessa a cui la loro organizzazione ha dato origine origine.

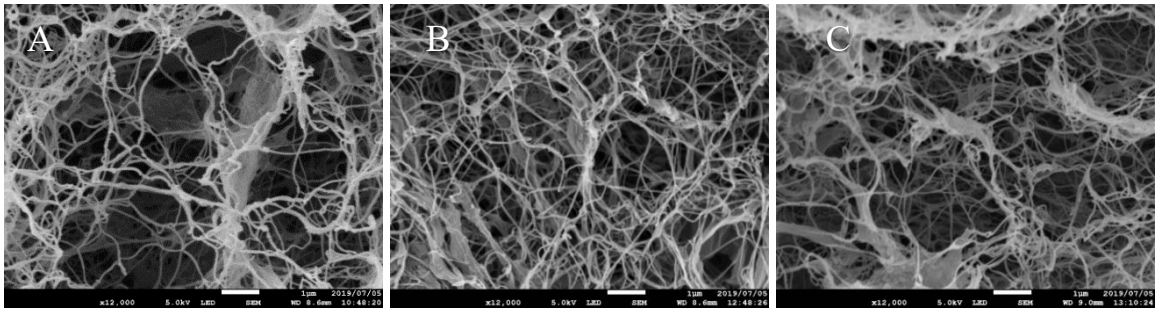


Fig. 3.4 Immagini SEM di (a) un idrogelo di collagene e di idrogeli caricati con (b) 0.5% e (c) 5% di ossido do grafene per confrontarne la morfologia interna

In particolare, si può notare come la densità di reticolazione risulti in generale aumentata in presenza di ossido di grafene, soprattutto nel caso di una concentrazione pari allo 0.5%. Seppur solo qualitativamente, questa analisi permette di confermare i risultati ottenuti dalla valutazione del contenuto d'acqua degli idrogeli, per i quali una percentuale di acqua del 91% e del 95,5%

erano state riscontrate nel caso di GO allo 0.5 e 5%, rispetto al 96% dell'idrogelo di collagene di riferimento.

3.7. Coltura cellulare

Gli idrogeli nanocompositi sono stati infine utilizzati come substrati per la coltura cellulare bidimensionale e come scaffold per quella tridimensionale allo scopo di valutare la loro applicabilità al campo dell'ingegneria tissutale, sia in termini di velocità di crescita cellulare sia in termini di citotossicità. Di seguito saranno presentati solo i risultati relativi alla coltura interna allo scaffold, che meglio riproduce l'ambiente fisiologico osservato nei tessuti.

Per questo esperimento, $5x10^4$ cellule appartenenti alla linea cellulare NIH3T3 sono state introdotte nella soluzione di collagene prima di essere sottoposta a gelificazione. Come per i precedenti esperimenti, un idrogelo di solo collagene e altri contenenti una concentrazione di GO pari allo 0.5, 2 e 5% sono stati realizzati. Questa volta gli idrogeli sono stati sintetizzati direttamente all'interno delle piastre per la coltura cellulare. Dopo essere stati sintetizzati, sono stati attentamente staccati dalle pareti delle piastre mediante l'uso di uno scalpello sterile, così da poter liberamente galleggiare sul mezzo di coltura. Dopo 10 giorni di incubazione a 37°C, con opportune ricorrenti sostituzioni del mezzo di coltura, i diametri degli idrogeli sono stati misurati con un calibro per valutare la contrazione dovuta alle forze di trazione esercitate dalle cellule in crescita. L'entità della contrazione è un parametro indicativo della rigidezza degli idrogeli l'uno rispetto all'altro.

Un confronto tra gli idrogeli dopo 10 giorni di incubazione è mostrato in fig. 3.5. Come si può osservare, l'idrogelo contenente lo 0.5% di GO si è contratto del 40%, rispetto al 47% dell'idrogelo di collagene. Ciò implica che in presenza di GO la rigidezza del gel è aumentata, aumentando di conseguenza la sua resistenza alle forze di trazione esercitate dalle cellule. Nel caso di GO al 2 e 5%, una contrazione dell'appena 7% è stata osservata. Tuttavia, questo dato non è da intendersi come uno spiccato aumento della rigidezza dei campioni, dal momento che l'analisi al microscopio dei gel ha mostrato un'assenza di crescita cellulare nel tempo.

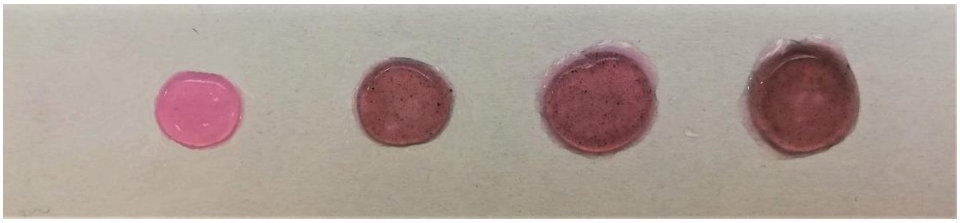


Fig. 3.5 Confronto del diametro degli idrogeli caricati con $5x10^4$ cellule del tipo NIH3T3 dopo dieci giorni di incubazione a 37°C. Si osservano, da sinistra, l'idrogelo di solo collagene e gli idrogeli contenenti GO allo 0.5, 2 e 5%.

Anche a conferma dei risultati ottenuti dagli esperimenti precedenti, è stato possibile concludere che un contenuto di GO pari allo 0.5% comporti il massimo aumento di rigidezza degli idrogeli, riscontrabile nella minor contrazione osservata rispetto agli idrogeli di solo collagene. Inoltre, per concentrazioni uguali e superiori al 2% w/w, l'ossido di grafene si è mostrato citotossico nei confronti delle cellule fibroblastiche.

4. Conclusioni

L'obiettivo di questo studio era quello di sintetizzare idrogeli di collagene caricati con ossido di grafene (GO) e di valutare gli effetti di quest'ultimo sulla struttura secondaria del collagene e sulle proprietà meccaniche e funzionali degli idrogeli che ne derivano. L'ossido, in concentrazioni comprese tra lo 0.05 e il 5% w/w, è stato utilizzato come rinforzante e reticolante fisico tra le catene di collagene in modo da evitare l'uso di agenti chimici che avrebbero potuto comprometterne l'applicazione nel campo della coltura cellulare.

Per gli idrogeli, sintetizzati tramite il protocollo fornito da *Nitta Gelatin Inc*® e opportunamente modificato ai nostri fini, il minor swelling, ossia la maggior densità di reticolazione, è stato osservato in corrispondenza di una concentrazione di ossido pari allo 0.5%, al di sopra del quale ha ripreso ad aumentare con l'aumento della concentrazione di ossido. La percentuale di acqua assorbita in questo caso è stata del 91%, rispetto al 96% degli idrogeli di solo collagene. Analisi della morfologia tramite SEM hanno confermato questo dato, evidenziando una struttura visibilmente più densa nel caso di una concentrazione di ossido pari allo 0.5% rispetto al 5% e, ancor più, rispetto ad un idrogelo di collagene di riferimento. Il modulo di immagazzinamento G' valutato tramite prove reologiche ha mostrato una diminuzione all'aumentare della concentrazione di GO, come osservato nel caso dello swelling, ad eccezione del caso di 5% GO in cui l'elevato contenuto di ossido ha fatto sì che la sua eccellente resistenza meccanica intrinseca prevalesse sulle proprietà della matrice di collagene. Una concentrazione di GO pari allo 0.5% rimane pertanto la più promettente, con un valore di G' di 180 Pa rispetto ai 120 Pa dell'idrogelo di collagene di riferimento. Valutazioni degli spettri di dicroismo circolare sono state effettuate per studiare l'effetto dell'ossido di grafene sulla struttura secondaria del collagene. Nonostante una diminuzione della percentuale di triple eliche all'aumentare della concentrazione di GO, la struttura secondaria è rimasta pressoché invariata, garantendo la non compromissione dell'attività richiesta affinché la proteina sia in grado di mantenere le sue funzioni biologiche. Misurazioni, effettuate dopo dieci giorni di incubazione, dei diametri degli idrogeli caricati con $5x10^4$ cellule, hanno riportato una contrazione dell'idrogelo caricato con 0.5% GO pari al 40%, rispetto al 47% del campione di riferimento. Questo risultato è indice di una maggior resistenza, e quindi rigidezza, del primo nei confronti delle forze di trazione esercitate dalle cellule. Concentrazioni del 2 e 5% hanno invece portato ad una contrazione del solo 7%, da attribuire però ad una mancata crescita delle cellule a causa dell'elevato contenuto di ossido, oltre il limite di tossicità.

Si conclude, pertanto, che idrogeli di collagene caricati con uno 0.5% di ossido di grafene costituiscano i migliori candidati come scaffold per la coltura cellulare e per la realizzazione di un tessuto equivalente. Concentrazioni più elevate di ossido compromettono il processo di fibrillogenesi che regola la formazione di idrogeli di collagene, oltre ad essere responsabili di citotossicità nei confronti delle cellule in coltura.

Index

1. Introduction and purposes

Tissue engineering is a relatively new field of regenerative medicine that refers to the practice of combining scaffolds, cells and biologically active molecules [I] to fabricate constructs that are structurally and functionally similar to biological tissues. It was officially defined as *"the application of principles and methods of engineering and life sciences toward the fundamental understanding of structure-function relationships in normal and pathological mammalian tissues and the development of biological substitutes to restore, maintain or improve tissue function"* in1988 [1].

Scaffold biomaterials have been widely recognized as the most promising candidates for the achievement of these purposes thanks to their highly porous structures able to guide the growth of new tissue [1]. Hydrogels, in particular, have been gaining particular attention as three-dimensional scaffolds due to their hydrophilic cross-linked network able to absorb a significant amount of water without dissolving [2]. This feature, along with their elasticity, flexibility and biocompatibility, increases their similarities to the extracellular matrix (ECM) of tissues. The ECM is the complex non-cellular micro-network, composed of a wide variety of structural proteins, that provides the biomechanical and biochemical support for the surrounding cells [3]. A strong stiffness-related influence of the ECM on cells behaviour has been shown to exist. Changes in the mechanical properties of the ECM, in fact, are responsible for the alteration of cellular morphology, differentiation, traction forces, adhesion and migration [4]. For this reason, the ability to tune the mechanical properties of hydrogels has become of primary importance for the development of tissue equivalents. Collagen is the most abundant protein of the ECM and, for this reason, collagen hydrogels provide an *in vivo*-like three-dimensional environment suitable for creating tissue equivalents and studying cell-matrix interactions [5]. Nevertheless, collagen hydrogels exhibit limited mechanical properties if compared to native collagen tissues. Chemical cross-linking of collagen is a common method to face this limit but the use of chemical agents usually induces cytotoxicity against the embedded cells. For this reason, physically cross-linked hydrogels are now considered an attractive alternative for cell culture applications.

Graphene oxide (GO) has been recently attracting attention in the biomedical field due to its unique properties such as large surface area, flexibility, high mechanical strength, high dispersibility, hydrophilicity, biocompatibility and ability to promote cell proliferation [6]. So far, its interaction with and its binding to proteins has been mainly studied for drug delivery or pH sensing applications, but little is known about its use as a reinforcing filler at low concentrations in collagen tissue equivalents.

In this study, physically cross-linked GO-collagen nanocomposite hydrogels at increasing concentrations of GO were fabricated by self-assembly of collagen at 37°C, without the use of chemical agents. The purpose was to investigate the effect of GO on the properties of the resulting hydrogels and to evaluate their suitability for cell culture in order to be used as tissue equivalents in the field of tissue engineering. The idea would be that of fabricating a tumoral-tissue-like *in vitro* model, capable of mimicking both structure and functionality of tumoral tissues, which would enable to understand the unknown mechanisms laying behind the development of untreatable cancer. Untreatable cancer is so defined because of the formation

of an impenetrable barrier around capillaries and veins that prevent drug nanoparticles from entering pathological tissues. By understanding the way this barrier is formed, a method to defeat it could be hopefully found.

This research embodies only the very first steps of this challenging and ambitious project and it lays the basis for several future experiments. In the present work, *Nitta Gelatin Inc*® protocol was modified according to our purposes and a new synthesizing method was studied for the fabrication of our GO-collagen nanocomposite hydrogels. A new method for the sterilization of GO was also established and its efficacy was tested through monolayer cell culture. The stiffness of these novel hydrogels was tested by rheologic measurements and by the evaluation of their contraction as a result of embedded cell culture. Cell growth speed was also qualitatively evaluated to study the effect of increasing concentrations of GO on the hydrogels stiffness. Circular dichroism (CD) spectroscopy was carried out on a collagen solution before and after addition of GO in order to investigate its effect on the secondary structure, and thus on the biological activity, of the protein. The evaluation of the hydrogels' water uptake at increasing concentrations of GO gave us information on their cross-linking density, which was also analysed and confirmed through SEM imaging.

An introductory chapter illustrating the state of the art behind collagen, collagen hydrogels, their use in tissue engineering, the study of cell-scaffold interactions, the properties of GO and its use in the biomedical field, and the GO-collagen hydrogels investigated so far, is presented. Follows a description of the materials and methods used throughout this research activity. Eventually, results will be discussed in detail before illustrating our final conclusions.

2. State of the art

In the following paragraphs, the most recent findings and advancements on collagen hydrogels and their use in the field of tissue engineering are presented.

2.1. Hydrogels for tissue engineering

Hydrogels are three-dimensional networks resulting from the cross-linking of hydrophilic polymers [7]. The nature of cross-linking junctions can be either physical or covalent and the hydrophilic polymers can be both natural or synthetic. As a consequence of the hydrophilicity of their network, hydrogels are capable of absorbing large amounts of water or biological fluids[2], giving rise to an extensively swollen, soft and compliant material.

When a hydrogel absorbs water, the first water molecules penetrating the network constitute the so called *primary attached water* and they interact with the highly polar and hydrophilic groups that build up the network. As a consequence of this first hydration, the hydrogel starts swelling leading to a decrease of the interactions existing among its hydrophobic regions. Once all of these interactions are abolished, *secondary attached water* can penetrate the network and bound to it as well. Eventually, when all polar, non-polar and ionic groups are saturated, the remaining free volume is filled with other water, defined as *free water* [11].

Despite being a necessary requirement, the hydrophilicity of the polymer constituting the hydrogel plays a marginal role on its ability to swell. The swelling ability of a hydrogel is, in fact, strongly dependent on its cross-linking density, which is also responsible for its final mechanical properties. In particular, cross-linking points prevent polymer chains from freely sliding with respect to one another. This means that the lower their concentration the higher the ability of the hydrogel to absorb water and swell. As a consequence, the swelling decreases as the cross-linking density increases. Cross-linking points can be the result of covalent bonds (chemical cross-linking) or hydrogen bonds, Van Der Waals forces, coulomb forces or *entanglements* (all related to physical cross-linking) [12] and they guarantee the formation of a network which, despite swelling, does not dissolve in water or biological fluids.

Based on the chemical or physical nature of the cross-linking junctions, hydrogels can be classified as chemically or physically cross-linked hydrogels respectively. Because of their irreversibility, chemical hydrogels are also defined as “permanent”. They can be the result of the polymerization of hydrophilic monomers through the use of cross-linking agents with low molecular weight, or they can be obtained by direct cross-linking of water soluble polymers [8]. The chemical reactions mainly involved in this process are condensation reactions, Michael addition, click reactions, enzymes-assisted reticulation and photoreticulation. Physical hydrogels, instead, result from molecular entanglements and/or secondary forces. For this reason they are often defined as “reversible”, thanks to their ability to dissolve when changes in the surrounding environmental conditions occur. Temperature, pH and ionic strength of the solution can in fact determine the reverse process. Due to significant differences in the amount of energy required to break chemical and physical bonds, chemically cross-linked hydrogels possess higher mechanical properties than their physical counterparts. On the other hand, though, chemicals frequently turn out to be cytotoxic against cells.

Hydrogels are classified also depending on the natural or synthetic origin of their constituting material. Natural hydrogels are usually fabricated from gelatin, collagen, alginate, fibrin or

hyaluronic acid and guarantee high compatibility and ability to mimic biological structures and functions. If compared to synthetic hydrogels, though, they turn out to be less easily reproducible because of the involvement of non-controllable natural processes. Synthetic hydrogels, in turn, could not be ideal for applications in the field of tissue engineering due to their toxicity-related risks, even if the biocompatibility of some polymers such as polyvinilachol (PVA)[10] and polyethylene glycol (PEG)[21] have been demonstrated.

Along with the high water content, hydrogels show other unique features such as softness, porosity, flexibility and biocompatibility [8]. The possibility of having all these properties combined in a single material has turned hydrogels into perfect candidates for the field of tissue engineering and drug delivery [13]. In fact, their ability to mimic natural living tissues opens up many opportunities for biomedical applications [8]. At this purpose, hydrogels must meet both physical and biological criteria to be able to promote tissue formation. The former is related to mechanical properties, which create and maintain a space for tissue development and depend on the original rigidity of polymer chains, on the type of cross-linking and on the cross-linking density of the hydrogels [13]. The latter instead refers to their biocompatibility and their ability to properly interact with cells to promote cell adhesion and growth.

Biomacromolecules have been recently attracting particular attention thanks to their ability to satisfy both the above mentioned requirements. If compared to synthetic polymers, in fact, their inherent biofunctionality and biocompatibility in biological environments [2] allow them to mimic the mechanisms of interaction of the extracellular matrix (ECM) with the surrounding cells, enabling the fabrication of tissue equivalents (TEs).

2.2. The extracellular matrix (ECM)

The extracellular matrix (ECM) is the complex non-cellular micro-network, composed of a wide variety of structural proteins, that provides the biomechanical and biochemical support for the surrounding cells [3]. The ECM is a reservoir of growth factors and cytokines for bioactive molecules and it is able to regulate their bioavailability and to establish their concentration gradients [19]. It responsible for angiogenesis and it allows cells to interact with the extracellular environment through signal pathways and receptors. It acts as a physical barrier, an anchorage site or a movement track for cell migration [20]. In order to fabricate tissue equivalents, it is then necessary to reproduce an ECM-like *in vitro* environment.

Being cells and tissues organized into three-dimensional architectures in our body, scaffold biomaterials like hydrogels have been extensively investigated as a 3D template for cell attachment and tissue formation [15] in order to mimic the extracellular matrix and its interaction with cells. Traditional two-dimensional cell culture systems, in fact, can highlight individual cell phenomena in homogeneous population of cells but they lead to growing cells in a non-physiological environment. As a consequence, they are not comparable to biological conditions. The three-dimensional structure of tissues is much more complex and it strongly affects the behaviour of cells *in vivo*. For this reason, cell-seeded scaffolds are able to better mimic the natural biophysical and biochemical microenvironment that promotes physiological functions of cells.

The three-dimensional architecture of the ECM is not the only parameter influencing cells behaviour. Also physical properties such as porosity, density and insolubility provide physical cues to cells [20]. Mechanical properties of the substrate have been shown to strongly affect cellular processes, which means that the mechanical properties of scaffolds directly influence

tissue formation. Stiffness of the ECM, in particular, affects cell morphology and acts on the mechanisms controlling cell adhesion differentiation and migration [16]. Stiffness has been demonstrated to increase from normal to tumour: normal tissues possess a Young's modulus comprised in the range from 0.5 to 2 kPa; tumoral tissues, instead, show a 10-20 fold increase of this value. For this reason, pathologic tissues strongly influence cells growth, facilitating cell migration, stimulating the formation of colonies with atypical structures and thus promoting tumour progression [15]. As a consequence, it is clear that the ability of mimicking a certain biological tissue is tightly related to the possibility of fabrication a scaffold having comparable stiffness.

Overall, there are several parameters determining the suitability of a scaffold for the fabrication of a tissue equivalent. Scaffolds should in fact meet many requirements in order for cells to create their own ECM microenvironment. From a mechanical point of view, for example, stiffness is not the only important parameter: scaffolds should also be resistant enough to maintain their structure and keep their inner space intact over the period of time required by cells to grow and create their environment. From a structural point of view, instead, one of the main issues is that of creating interconnected pore structures that allow an homogeneous migration of cells. A porous structure also facilitates cell processes, being cell attachment and growth promoted by wide available space and large surface area. In terms of biological properties, eventually, they should be able to mimic the ECM and its signalling molecules and for this reason natural biopolymers such as collagen are used [15].

2.3. Collagen and its structure

Among biomacromolecules, such as polysaccharides, nucleic acids, polypeptides and proteins, collagen has gained a particular interest in the field of tissue engineering. Collagen is a structural protein and it is the most abundant component in all animals [14]. As a consequence, it is also the main component of the ECM.

Twenty-eight different types of collagen deriving from the assembly of forty-six distinct collagen chains have been identified so far. Each type can be categorized into fibril-forming collagens (e.g., types I, II, III), network forming collagens (e.g., type IV), fibril-associated collagens with interruptions in their helices (e.g., types IX, XII) and others (e.g., types VI) [19].

Collagen type I is the most abundant collagen of the human body and it is a fibril-forming collagen that consists of amino acids bound together to form a triple helix. In particular, this triple helical structure results from the assembly of three left-handed amino acid sequences of polyproline II (PPII). All peptide bonds of PPII are in the *trans* conformation [14] and a proper folding of the chains is guaranteed by the presence of a glycine residue in every third position in the polypeptide chain, as illustrated by M. D. Shoulders et.al [fig. 2.1]. The three PPII chains are then twisted around one another in a rope-like manner to produce the overall tightly packed triple-helical form of the molecule [II]. The chains interact through a single interchain hydrogen bonding per triplet [14], which is responsible for the overall resistance of the resulting collagen molecule. Thanks to the presence of the amino group (NH) of glycine residues which can form a peptide bond with the carbonyl (C=O) group of adjacent residues, an hydrogen bonding network around each PPII chain is formed, giving rise to an hydration sphere. A collagen molecule is approximately 300 nm long and 2 nm in diameter [II].

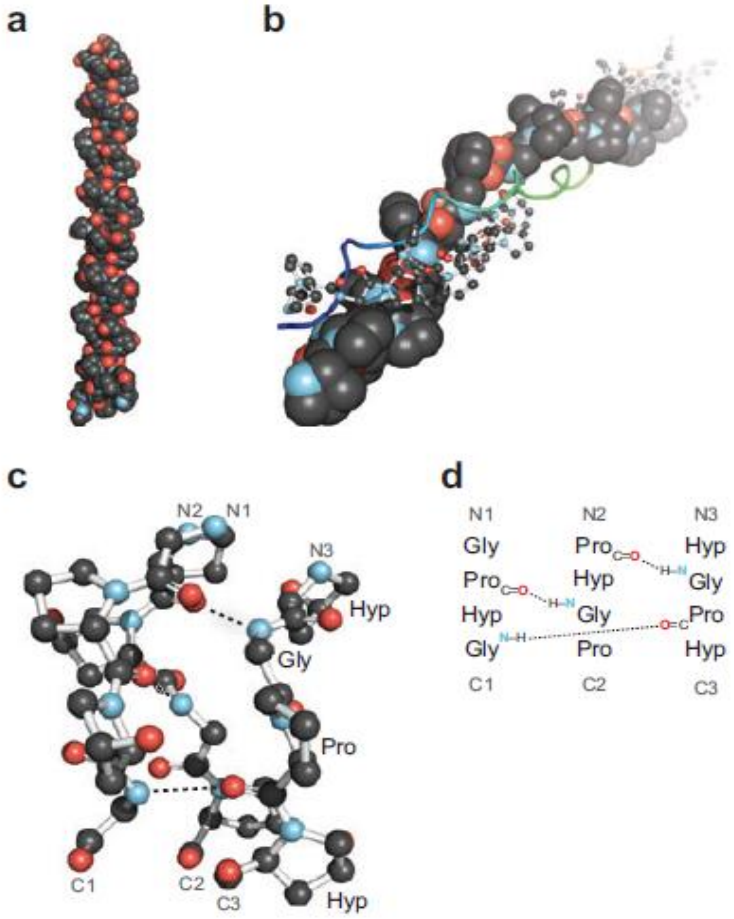


Fig. 2.1 Overview of the collagen triple helix. (*a*) High-resolution crystal structure of a collagen triple helix. (*b*) View of a triple helix with the three strands depicted in space-filling, ball-and-stick, and ribbon representation. (*c*) Ball-and-stick image of a segment of collagen triple helix, highlighting the ladder of interstrand hydrogen bonds. (*d*) Stagger of the three strands in the segment in panel *c*. [14]

It is important to underline that only native collagen consists of a single triple helical domain, since other types of collagen are usually composed of multiple helical domains. In this case, each domain constitutes only a fraction of the mass of the collagen molecule instead of its 95%.

The triple helical structure of collagen is responsible for many of the characteristics that make it a good candidate for the fabrication of scaffold materials in tissue engineering, such as thermal stability, mechanical strength and ability to interact with other macromolecules [14]. For example, the amide-amide hydrogen bond within the triple helix has been estimated to have a strength of around ΔG° = -2.0 kcal/mol, which determines the mechanical properties of the collagen molecule. Similarly, it has been noticed that mutations on the triple helical structure are responsible for many collagen-related diseases, especially when glycine residues get replaced by amino acids.

2.4. Fibrillogenesis of collagen

In native tissues, triple helices of collagen self-assemble into fibrils according to a process known as *fibrillogenesis*. These fibrils are further assembled into fibers under the guide of cells. The resulting fibrils are 10-300 nm in diameter and can be hundreds of microns long, while fibers can be several microns in diameter and millimetres to centimetres in length [18]. Fibrils and fibers are responsible for the stiffness and the structural support of tissues. Moreover, they influence cell adhesion, growth, migration and differentiation, all biological functions based on the interaction of collagen with cells or other proteins of the ECM [19]. In vivo, fibrils and fibers are stabilized via covalent cross-links.

In vitro collagen tissue equivalents, though, possess significantly lower mechanical properties than the corresponding native tissues. Despite being extracted from biological tissues, in fact, collagen can only partially reorganize into its original architecture and reproduce it. Also from a chemical point of view, its composition is not intact due to the extraction processes involved [2]. Collagen isolation ex-vivo, in fact, leads to the breakage of native hydrogen and covalent bonds [2]. This explains why the resulting collagen hydrogels show poor mechanical properties if compared to the corresponding biological tissues: helices reorganize through the formation of physical interactions and entanglements, while original covalent bonds are not restored. To face this limit, physical and chemical cross-linking, as well as modified gelling strategies, are commonly used, even if the resulting stabilization of the collagen structures is still insufficient. The morphologic organization of the resulting hydrogels is also affected.

Extracted collagen is commonly stocked in acidic conditions (pH=3) and low temperatures (4°C) in order to avoid its undesired *fibrillogenesis*, i.e. the self-assembly of its helices into fibrils that occurs also in native tissues. For a pH value below 3, collagen triple helices get disrupted by the high concentrations of H^+ ions found in solution, which are responsible for the generation of repulsive forces within the strands. Above pH 3, instead, dispersion decreases with the increasing value of pH. A pH equal to 3 then guarantees the best dispersing ability of the protein. When desired, fibrillogenesis can be induced by exposing the triple helical collagen to physiological conditions: upon neutralization of the solution through the use of a reconstitution buffer, triple helices self-assemble into fibrils giving rise to a collagen hydrogel.

Various factors influence the self-assembly of collagen, especially temperature, pH and ionic strength. Collagen hydrogels are usually fabricated at the physiological temperature of 37°C, even if self-assembly starts as soon as the solution is neutralized. Most research groups keep the mixing tubes on ice to delay self-assembly, even if fibrillogenesis at low temperatures has been recently reported to be particularly successful by Yang. et al. The most desirable pore size for cellular proliferation was in fact obtained at temperatures down 4°C [22], while room temperature led to more stable hydrogels over time [23]. Because kinetics is temperature-dependent, higher temperatures lead to a more rapid self-assembly of collagen helices resulting in thinner fibers (combination of a lower number of fibrils) and a less ordered structure [4]. Overall structural, transport and mechanical properties are altered by temperature. Similar effects are observed by controlling the gelation pH. Physiological pH in the range from 7.4 to 8.4 is commonly used in order not to affect cell viability. As well as temperature, an increase in the pH value promotes electrostatic interactions and fiber nucleation, accelerating fibrillogenesis and producing fibers with reduced diameter and networks with smaller pore sizes [22]. When considering the effect of ionic strength, instead, an opposite trend is observed: pore size and fiber diameter decrease with the decreasing ionic strength.

2.5. Collagen hydrogels

The purpose of tissue engineering it to fabricate *in vitro* models capable of structurally and functionally mimicking biological tissues. Since function is dependent on structure [1], the ability to reproduce the micro-architectural structure of the extracellular matrix is of primary importance to achieve this goal.

As previously reported, collagen is the most abundant protein in the ECM. For this reason, collagen hydrogels provide an *in vivo*-like three-dimensional environment suitable for creating tissue equivalents and studying cell-matrix interactions [5]. Being a natural component of our body, in fact, it meets many of the biological design parameters required for tissue engineering applications, as it is composed of specific combination of amino acids that can be recognized by cells [17]. Its fibrous architecture provides a favorable microenvironment for cell attachment, binding of growth factors and regulation of cell signal and behaviour [1].

Collagen hydrogels have been attracting particular attention also thanks to their quick and simple preparation. As explained in the previous paragraph, collagen monomers solubilized in acidic conditions and kept at low temperatures are neutralized and warmed up to 37°C to induce gelation. Monomers assemble into fibrils that can further aggregate into fibers through an entropy-driven self-assembly process that is strongly affected by the nature of the collagen monomer and is sensitive to temperature, ionic strength and pH [1]. These parameters influence the formation of fibrils and consequently affect cellular response.

The packing of collagen fibrils in self-assembled hydrogels is strongly influenced by the ratio of non-helical regions present in solution before gelation, even if they can be easily removed through the digestion operated by enzymes. The concentration of collagen solution is another factor determining the final structure and properties of the hydrogel. The density of the network increases with the increasing concentration of collagen, with a corresponding increase in the hydrogel's stiffness. Through the analysis of biological tissue it was in fact found that cancerous tissues contain 9 to 45 mg/ml of collagen in the interstitium while normal tissues contain significantly less [22], and the stiffness of tumoral tissues is 10-20 times that of non-pathologic tissues. As a consequence, the concentration of collagen is also responsible for cellular precesses.

In order to fabricate tissue equivalents, collagen hydrogels are cell-seeded before undergoing gelation in order to include cells in their network. This way, cells can grow in a 3D environment similar to that found in biological systems until they give rise to an *in vitro* tissue model. The presence of cells leads to cell-driven remodelling processes of the hosting hydrogel, resulting in compaction and consolidation [1].

When cells like fibroblasts are added to collagen hydrogels they usually display a rounded shape. After migration through the gel and adhesion to the network, a bipolar or stellate morphology is observed instead, depending on the presence or absence of constraints to the hydrogel respectively. An unchanged morphology over time is undesired because it is symptom of dead cells. After adhesion has occurred, compaction and stiffening of the hydrogel starts as a consequence of the contractile forces exerted by the growing cells [fig. 2.2]. Remodelling of the hydrogel is due to the rearrangement of existing fibrils, which are brought together under the action of growing cells. These fibrils bind together through non-covalent bonds and the result is a stabilization of the hydrogel's network. The rate of compaction depends on the resistance of the collagen network and on the number of embedded cells. Compaction of the hydrogel proceeds only if the traction forces exceed the resistance of the collagen matrix [1]. When this happens, fibrils rearrange on a large scale and lead to a decrease in pore size and an

increase in network density. Cell-driven contraction result in a significant volume reduction, sometimes also up to 90%.

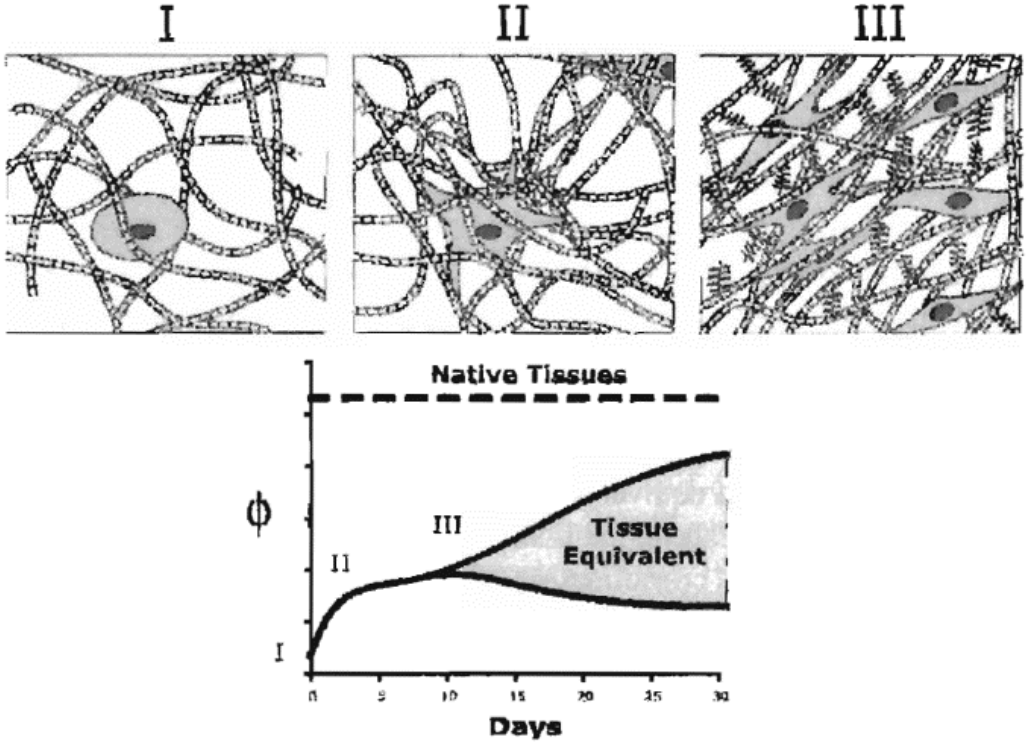


Fig. 2.2 Gel compaction and remodelling: change in volume fraction over time. (I) Initially, (II) in a matters of hours, (III) over the next few weeks [1].

The compaction of the hydrogel is strongly affected by the presence or absence of physical constraints, such as adherent surfaces or embedded obstacles, that can impede the propagation of the traction forces exerted by cells through the network. When hydrogels are attached to the walls and base of the culture dish, compaction occurs through the thickness while the original diameter is preserved. In particular, because fibrils are not free to translate in presence of adherent surfaces, they align with the tension exerted by cells, giving rise to peculiar alignment patterns depending on where constraints are located [1]. Free-floating hydrogels, instead, result in a significant contraction of both thickness and diameter and fibrils give rise to a random isotropic network. In general, compaction occurs if the hydrogel is able to sustain tension, since disruption of the network would stop the propagation of traction forces through it.

The overall rate of contraction is strongly influenced by the initial concentration of collagen and cells. By considering an unconstrained free-floating hydrogel, the higher the initial cell number the greater the total contraction and the higher the contraction rate at which it occurred. The same behaviour is observed when the initial concentration of collagen is decreased. Cells and collagen concentrations are coupled, so the lowest compaction is observed in low-cell high-collagen-density hydrogels, while the greatest is observed in high-cell low-collagen-density hydrogels [1]. Nevertheless, above a threshold cell number contraction is no longer affected due to a lack of binding sites. Overall, collagen concentration as a more significant influence than cell concentration.

Despite their ability to remodel and to mimic the ECM, collagen hydrogels still present significant limits due to their poor mechanical properties. The breakage of the original bonds during extraction from natural tissues leads to the formation of hydrogels with a final density

of collagen which is much lower than that observed in biological tissues. As widely explained, this strongly influences cell adhesion, growth and differentiation.

For this reason, the necessity of reinforcing collagen hydrogels has become of primary importance in recent years. Since single materials are hardly capable of mimicking both physical and biological properties of native tissues, hybrid materials with multiple components addressing different requirements are now widely used to fabricate tissue equivalents [24]. The use of fillers allows to act on the mechanical properties of collagen without the use of chemical cross-linking agents that could be harmful for embedded cells.

2.6. Graphene oxide (GO) for tissue engineering

Graphene and its derivatives have been intensely studied for their unique fascinating properties such as large surface area, high aspect ratio and outstanding mechanical, thermal, electrical as well as optical properties [6,25]. Recently, they have also been explored as biocompatible nanomaterials for drug delivery, biosensing and cell culture and growth[28] thanks to their high purity, good bio-functionalizability, high drug loading capacity and capability of easy cell membrane penetration [6].

Graphene oxide (GO) is the oxidized form of graphene [fig. 2.3], a two-dimensional one-atom thick layer of sp^2 carbon atoms [25] packed into a honeycomb lattice.

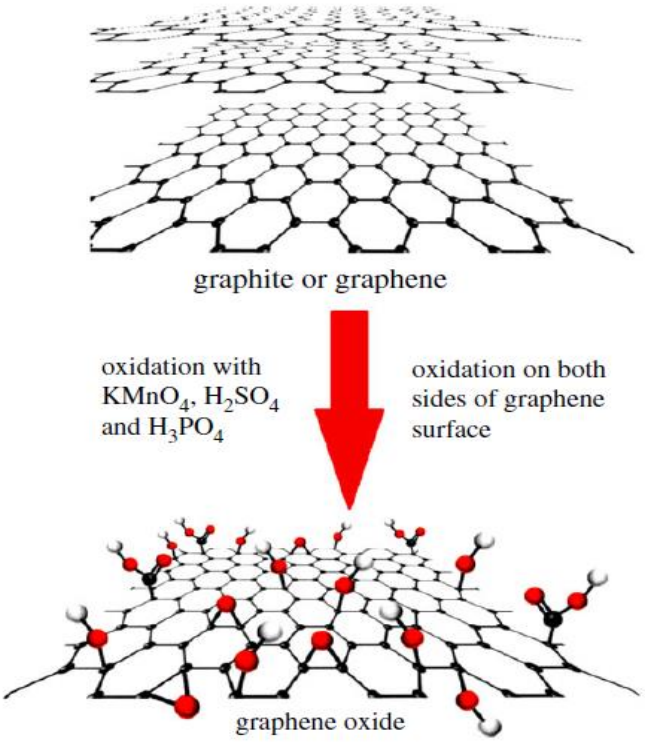


Fig. 2.3 Schematic representation of the formation of graphene oxide (GO) from graphene (or graphite) via Hummers method [6]

Graphene is obtained from the exfoliation of graphite and it is hydrophobic and non-dispersible in water. As the oxidized form of graphene, GO is instead an aqueous dispersible and colloidal stable [26] material thanks to the presence of carbonyl, hydroxyl and epoxydic functional groups on its surface. These oxygen-containing groups also provide anchor sites for binding with polymers [27], including natural polymers like collagen. GO interacts with proteins via chemical bonding or via physical adsorption through π–π stacking interactions and hydrogen bonding, which occur between the nitrogen/oxygen containing groups of GO and the oxygen functional groups of proteins.

In order to achieve good biomedical applications, GO should be properly prepared and functionalized [6]. Oxidation of graphite was at first approached as a method to achieve graphite exfoliation on a large scale and obtain purely single-layer graphene. The resulting GO is of great interest due to its low cost, easy access and ability to be converted into graphene. In 1958, Hummers reported a faster and safer method for the synthesis of GO by using $KMnO_4$ and $NaNO_3$ as catalysts in concentrated H_2SO_4 [29]. In particular, 3 wt equivalents of potassium permanganate (KMnO4) and 0.5 wt equivalents of sodium nitrate ($NaNO_3$) were used to convert 1 wt equivalent of graphite to graphite oxide [31]. The reaction was carried out in concentrated H_2SO_4 first at 66°C and then down to 0°C, while stirring. This method allows to still maintain a relatively high C/O ratio despite oxidation of the carbon structure. It is important to underline that GO is a non-stoichiometric compound of carbon, oxygen and hydrogen in various ratios that depend on the processing methodology [29]. Over time, the *Hummers method* has been modified several times in order to make the process more efficient and environmentally friendly.

GO has been recently used in the field of cell culture and tissue engineering because of its very good adhesion and proliferation properties, excellent gene transfection efficacy and high ability to promote differentiation of stem cells [6]. The ability to enhance biological functions is related to physicochemical factors including large surface area, nanoscale roughness, the presence of pendant groups, hydrophilic nature and high water retention ability [32]. Its surface has also been reported to have a size-related antibacterial effect, even if its ability to inhibit bacterial growth is controversial. Therefore, modification of a scaffold using GO nanosheets should promote biological responses and tissue-reforming activity [30], as well as mechanical properties enhancement. Recent studies have in fact shown the ability of GO to reinforce the network of scaffold biomaterials and to biologically activate natural polymers such as collagen thanks to presence of functional groups. In the first case, these groups promote interfacial interaction with the matrix enabling stress transfer [32], in the second they closely mimic the properties of native bones and induce biomimetic mineralization [27].

The ability of GO to promote biological responses is a proof of its biocompatibility. Nevertheless, biological applications also require previous studies on its cytotoxicity, which has been widely yet not fully investigated. So far, cytotoxicity of GO was found to be concentration- and time-dependent [33] as well as dependent on size and layer [26]. In particular, doses less than 20 μg/mL did not exhibit toxicity to human fibroblast cells and animals, while doses of more than 50 μg/mL exhibited high cytotoxicity leading to apoptosis and decreased cell adhesion [33]. The mechanism responsible for GO-related toxicity is its ability to break membranes and enter into cytoplasm and nucleus, as well as to induce oxidative stress in cells in a concentration-dependent manner [34].

Overall, GO has the ability to promote cell functions and to mechanically reinforce scaffold biomaterials thanks to the presence of oxygen-containing functional groups on its surface. Nevertheless, its size-, concentration- and time- dependent cytotoxicity should be carefully taken into account for applications in the field of tissue engineering.

2.7. GO-reinforced collagen hydrogels

Several GO-reinforced polymer hydrogels in which monomers were *in situ* polymerized and chemically cross-linked with GO, which acted as a reinforcing filler, have been reported.

However, studies on nanocomposite hydrogels based on GO and polycationic natural polymers such as chitosan, collagen and gelatin, the partially-denatured form of collagen, are limited [25]. Physically and mostly chemically cross-linked hydrogels have been investigated in this field.

Han et al. reported self-assembled GO-chitosan hydrogels which showed a storage modulus of 0.7 kPa and self-healing properties. Faghihi et al. instead, fabricated chemically cross-linked GO-poly(acrylic acid)-gelatin nanocomposite hydrogels that exhibited a tensile strength of 150-250 kPa, even if the cross-linker turned out to be cytotoxic [25].

Based on previous studies which reported the ability of incorporated GO to enhance the mechanical properties of scaffolds, S. Kang et al. [34] investigated the mechanical properties and osteogenic differentiation of 3D collagen sponges covalently conjugated with stiff GO flakes through the use of 1-ehtyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) to activate the carboxyl groups of GO. The GO conjugation did not cause cytotoxicity, it stimulated cell adhesion and increased the scaffold stiffness by 3-fold, making it comparable to that of pre-mineralized collagenous bone matrix. Scaffolds were obtained by immersing the previously fabricated collagen sponges into a GO/EDC solution.

Piao and Chen fabricated novel GO-gelatin nanocomposite hydrogels both self-assembled at 37°C [25] and chemically cross-linked at 95°C as a result of the reduction of GO by gelatin [35]. The highest storage modulus of rGO–gelatin hydrogels was 172.3 kPa, 50% higher than that of its physically cross-linked counterpart. Hydrogels were obtained from a solution containing 10 mg/ml GO and 2, 5 or 10 mg/ml gelatin.

3. Materials

Nitta Gelatin Inc.® Collagen type I-A 3mg/ml, *NiSiNa Materials* graphene oxide (GO), *Nitta Gelatin Inc.*® reconstitution buffer, Dulbecco's modified Eagle Medium (DMEM) 5x, EtOH 85%, HCl 10^{-3}M, *Sterlab Scientific*® PES sterile syringe filters 0.22µm pore size, NIH3T3 cell line, *Corning*™ 24-well culture dishes treated and non-treated.

4. Methods

4.1. Preparation of the GO-collagen nanocomposite hydrogels

4.1.1. Optical microscope analysis and exfoliation of the GO suspension

A rough check of the overall size distribution of the oxides' flakes in suspension was conducted by using an optic microscope at 10x magnification. A narrow size distribution is in fact the condition required to guarantee the fabrication of a successful and especially repeatable nanocomposite. Since inhomogeneities were observed, presumably due to aggregation of the graphene oxide flakes, the suspension was magnetically stirred for 2 hours and then subjected to sonication for 1 hour to achieve exfoliation of the aggregated flakes. The resulting suspension was once again observed at the microscope in order to confirm that size homogeneity had been achieved. For better clarity in terms of size, non-exfoliated and exfoliated GO could be defined as large and small GO respectively.

4.1.2. Sterilization of the GO suspension

Sterilization of the GO suspension was required to guarantee the fabrication of sterile hydrogels for cell culture purposes. *Nitta Collagen Inc.*® collagen type I-A and reconstitution buffer were already purchased as specific products for cell culture.

As a preparatory step, DMEM 5x and EtOH 85% were filtered into sterile vessels by using *Sterlab Scientific*® PES sterile syringe filters with a pore size of 0.22µm. Being the graphene oxide suspension dense, it was diluted from 10 mg/ml down to 1 mg/ml by adding sterile water to facilitate the following step, centrifugation.

Sterilization was performed through multiple centrifugations and resuspensions in EtOH 85%. Firstly, the non-sterile GO suspension was centrifugated and the supernatant was removed. Then, after the addition of EtOH 85%, the suspension was moved to a new sterile vessel and subjected to centrifugation, at the end of which the supernatant was removed. This sterilizing step through the use EtOH 85% was repeated three times. After that, the oxide was washed twice with DMEM 5x before a final resuspension in DMEM 5x up to the initial volume of the suspensions, in order to preserve the initial concentration.

Centrifugations were performed at 14000 rpm and 4°C for 30 minutes. Each centrifugation step was followed by the removal of the supernatant and the addition of a new suspension medium. These operations were performed inside the clean bench in order to guarantee sterility. The amount of suspension medium added at each step was as enough as to guarantee a proper sterilization or washing of the oxide. Only the amount of DMEM 5x for the final resuspension was significant to preserve the initial concentration of 1mg/ml.

Resuspensions were achieved by gently mixing with a pipette. The final resuspension in DMEM 5x is a preparatory step for the following synthesis of the GO-charged collagen hydrogels, which will be used as matrices for cell culture.

4.1.3. Adjustment of the final concentration of the sterilized GO suspension

Sterilization of the suspension led to a small loss of oxide at each removal of the supernatant, due to the hydrophilicity of GO. Being aware of this, TGA analysis of the final suspension was performed in order to evaluate the µg of oxide contained in a given amount of sterile suspension.

Measurements were taken on an aluminium pan and in nitrogen/O_2 atmosphere, in the range from 30°C to 630°C at a speed of 10°C/min. Before cooling down back to 30°C at the same speed, an isothermal step at 630°C was carried out for 10 mins in order to guarantee a complete pyrolysis of GO.

We analysed 30µl of suspension. In case of a suspension at the concentration of 1 mg/ml like the initial one, the loss of weight related to the pyrolysis of the oxide should have corresponded to 30µm. In our case, a lower pyrolysis-related loss of weight was expected due to the above mentioned reasons.

After the measurements, adjustments to the suspension have been made according to the amount of GO resulting from the loss of weight related to its pyrolysis. This phenomenon has been reported to start around 500°C and be over at around 600°C [III].

At the end of this process, the concentration was adjusted and two GO suspension, 1mg/ml and 0.1mg/ml, were prepared to be used for the synthesis of sterile hydrogels for cell culture.

3.1.4 Synthesis of the hydrogels

GO-collagen nanocomposite hydrogels with an increasing amount of GO were prepared by following the gelation procedure provided by Nitta Gelatin Incorporation©. The whole procedure was conducted while keeping the solutions in ice to prevent an undesired early gelation of collagen.

In case of simple collagen hydrogels non-charged with GO, Collagen Cell Matrix Type I-A 3mg/ml (A) was gently mixed with 5x concentrated DMEM (B) and eventually neutralized with the reconstitution buffer (C) applying the mixture ratio A:B:C=7:2:1. This solution is called reconstituted collagen solution. At both steps, mixing should be conducted carefully and until having reached a good homogeneity.

We prepared 250µl of reconstituted collagen solution, which included 175µl of collagen, 50µl of DMEM 5x and 25µl of buffer solution according to the above mentioned ratio. The final concentration of collagen in the gel was 2,1mg/ml. A silicone mold with a dimeter of 8 mm was placed on a non-treated cell culture dish. The reconstituted collagen solution was then transferred into the mold and the cell culture dish moved to an oven at 37°C for 30 mins.

In order to charge the collagen hydrogels with GO, the procedure was slightly modified according to our purposes without affecting the final concentration of collagen in the gels. This allowed us to make comparisons between the non-charged and GO-charged collagen hydrogels.

Seven gels were prepared at an increasing concentration of oxide: 0.05% ,0.1%, 0.2%, 0.5%, 1%, 2%, 5%. The percentage has to be intended as a w/w% of the oxide with respect to the mg of collagen contained in the amount of collagen solution used for the fabrication of the gels. A GO suspension at the concentration of 0,1mg/ml was used for the hydrogels ranging from 0,05% to 0,2% w/w% of GO, while a 1mg/ml suspension was used for the remaining higher concentrations. This allowed us to keep the required amount of GO suspension lower than that of DMEM 5x even at higher concentrations of GO, i.e. to guarantee the same ratio among the

constituents. In fact, in order to maintain the A:B:C=7:2:1 ratio, the amounts of GO suspension were subtracted to the 50µl of DMEM 5x previously calculated for the non-charged collagen hydrogel.

As a consequence, a new procedure was established as follows: while keeping the tube on ice, DMEM 5x and GO 0,1mg/ml or 1mg/ml (depending on the desired concentration) were mixed and a final mixture of 50µl was obtained. Collagen Cell Matrix Type I-A was added and well mixed. Eventually, the buffer solution was added and well mixed. The final mixture was transferred to an oven at 37°C for half an hour as already mentioned before. GO-charged collagen hydrogels were prepared by using both exfoliated and non-exfoliated GO.

6.1 Characterization of the hydrogels and of the interaction between collagen and GO

3.2.1 Water uptake

The amount of water retained by a hydrogel is an indicator for its cross-linking density. Empty glass vessels were dried up under vacuum at 100°C overnight and weighed right after having been taken out of the vacuum chamber. Meanwhile, hydrogels were prepared according to the previously described procedure, and then immediately placed inside the vessels and weighed. Eventually, they were let dry overnight at room temperature under vacuum and weighed one last time in dried conditions. The water uptake was evaluated according to eq. 3.1:

$$\%water = \frac{Ww-Wd}{Ww-W0}\text{x}100 \qquad (3.1)$$

where w_0 is the weight of the empty vessel after drying, w_w the weight of the vessel containing the wet hydrogel, w_d the weight of the same vessel containing the dried hydrogel.

Comparisons were made between non-charged collagen hydrogels and hydrogels charged with exfoliated GO and non-exfoliated GO.

3.2.2 CD spectroscopy

CD spectroscopy measurements were taken to evaluate the effect of GO on the secondary structure of type I collagen. In doing so, the initial 3mg/ml collagen solution was diluted down to 0,3 mg/ml to prevent saturation of the signal during the measurements. Dilution was performed by adding a proper amount of HCl 10^{-3}M to keep the solution at a pH equal to 3 as the original one. 300µl of diluted collagen solution were used for each measurement. In order to study the interaction in the same conditions as the ones occurring during the formation of the hydrogels, DMEM 5x and buffer were also added to the diluted collagen solution to neutralize it. Both solutions were previously filtered using a 0.22µm PES filter to remove any undesired body, like dust, that might have affected the absorbance. Solutions rich in ions might have also affected the signal as well. For this reason, a concentration of DMEM 5x and buffer lower than the one deriving from the ratio 7:2:1 was used, in order to reduce the noise at its minimum but still guaranteeing a final pH equal to 7. By using a universal pH indicator, it was in fact found

that 300µl of collagen solution were neutralized upon addition of 0.4µl of DMEM 5x and 2µl of buffer. GO at concentrations of 0.5%, 2% and 5% was added to this neutralized solution. Unlike DMEM 5x and buffer, the ratio between GO and collagen was maintained. A GO suspension at 0,1mg/ml was used. The amount of GO suspension to be added was in this case evaluated with respect to 300µl of collagen solution instead of 175µl as reported for the gels.

For GO at each concentration, measurements were taken both immediately after the addition of the oxide and after 4 hours of incubation, and with both exfoliated and non-exfoliated GO. A dynamic effect of graphene oxide on the secondary structure of siRNA over time was in fact reported by Nishina et.al [37], and our purpose was to investigate whether the same reasonings could be applied to the interaction between graphene oxide and type I collagen or not. In the same article it was also reported that small GO tends to intercalate between the double strand causing destabilization, while large GO interacts with the RNA groove leading to a decrease of the winding angle of the double helix. Collagen shows a triple helical structure and for this reason there might be some similarities in its interaction with GO of different sizes.

To sum up, samples included:

- 300 µl collagen 0,3mg/ml
- 0.4 µl DMEM 5x
- 2 µl buffer
- GO suspension, both exfoliated and non-exfoliated, at a concentration of 0,1mg/ml

The above mentioned samples were tested according to the following experimental conditions:

- Wavelength range: 250-290 nm
- Data interval: 1 nm
- Scanning mode: continuous
- Response: 4 sec
- Band width: 1 nm
- Number of measurements: 10

The cuvette had an internal path length equal to 1 mm.

Measurements were taken at 4°C.

Ellipticity measurements were corrected for the cuvette baseline and the spectrum of the buffer and DMEM 5x solution was subtracted from each sample's spectrum as well.

3.2.3 Rheology

Oscillatory shear evaluations were performed at 25°C with a parallel plate geometry. Rheologic measurements were carried out in strain controlled conditions over an angular frequency range from 1 to 100 rad/s under a fixed strain of 5%, in the linear viscoelastic region previously determined by dynamic strain sweep tests. The gap between the two parallel plates was fixed to 1 mm. Because of our hydrogels being particularly slippery and hard to keep in position on the plate, sandpaper was attached to both parallel plates of the rheometer to facilitate the measurements. One collagen hydrogel and GO-collagen hydrogels at increasing concentrations of GO, both exfoliated and non-exfoliated, equal to 0.5, 2 and 5% were tested.

The storage modulus G', loss modulus G'' and loss factor δ were evaluated to determine the viscoelastic properties of the nanocomposite hydrogels.

3.2.4 SEM

Scanning electron microscopy (SEM) was carried out at 5 kV on dehydrated samples. One collagen hydrogel and two GO-charged collagen hydrogels, at a concentration of GO equal to 0.5% and 5%, were prepared according to the experimental procedure previously displayed.

The samples for SEM were prepared by fixation of the hydrogels with glutaraldehyde followed by serial dehydration with ethanol and freeze drying. A first immersion in glutaraldehyde 2.5% for 1 hour at 4°C to stabilize and fix the structure of collagen was performed, followed by an immersion in 1x PBS for 1 hour at 4°C to wash glutaraldehyde away. After that, the hydrogels were immersed in solutions at increasing concentrations of EtOH (60%, 80%, 90%, 95%, 99,5%, 100%). Each immersion was carried out for 1 hour at 4°C, from the solution with the lowest to the one with the highest concentration of ethanol. These multiple immersions in EtOH aimed to progressively dehydrate the hydrogels' 3D networks. At this purpose, the immersion in 100% EtOH was performed twice.

A final immersion in tertiary-butyl-alcohol for 1 hour at RT was carried out to exchange the solvent. After that, the gels were moved into new t-butyl-alcohol before freezing it down in a refrigerator at 4°C, as a preparatory step for freeze-drying.

The hydrogels were freeze-dried overnight under vacuum at 10Pa to obtain perfectly dried samples for SEM.

The samples were then carefully cut and mounted on SEM sample stages using carbon tape. The fracture surfaces were eventually sputter coated with Au in order to guarantee the conductivity required to observe samples under the electron microscope.

Images were taken at magnification of 12000x.

3.3 Cell culture

Monolayer and embedded cell culture were performed to evaluate the suitability of GO-collagen hydrogels for applications in the field of cell culture and tissue engineering, both in terms of cell-growth speed and cytotoxicity. For the following cell culture experiments, only non-exfoliated GO was used because of lack of time, which would have instead been required to repeat the analysis in a different condition. As a previous step to both 2D and 3D experiments, fibroblasts belonging to the NIH3T3 cell line were expanded to guarantee the presence of a proper amount of cells. Re-plating of cells was performed up to 5 times not to affect their growth speed and always before confluency was reached. Confluency is defined as the percentage of the culture vessel surface area that appears covered by a layer of cells when observed under a microscope.

3.3.1 2D cell culture

Monolayer cell culture was performed with the main aim to evaluate the efficacy of the sterilization of GO by EtOH 85%. The experiment is also a method to qualitatively analyse the growth of cells on hydrogels with different stiffness. Collagen hydrogel and GO-collagen nanocomposite hydrogels at concentrations of GO equal to 0.5, 2 and 5% were prepared inside 24-well treated cell culture dishes according to the same procedure displayed for experiments

previously performed to characterize the hydrogels. After 30 minutes of incubation at 37°C to allow for gelation, $5x10^3$ NIH3T3 cells were deposited on each hydrogel and 400 µl of culture medium (DMEM) were added [fig. 3.1] to guarantee the formation of a 2mm-thick layer commonly required to maintain cells in culture. Culture medium was changed every 2-3 days after having carefully washed with PBS 1x. Hydrogels were observed under an optic microscope at 10x magnification to register the growth of cells at day 1, 3, 5 and 7, considering as 0 the day of the experimental setup. 3 samples of each type were prepared for each day, for a total of 48 samples, to allow for the quantitative evaluation of cell growth through the analysis of their fluorescence. The fluorescence assay consists in preparing pellets an containing increasing definite number of cells to evaluate the fluorescence intensity corresponding to each specific amount. Interpolation of intensity against number of cells gives rise to a linear graph that can be used as a reference to calculate the number of cells grown on each hydrogels. After having evaluated the fluorescence of each sample, in fact, the corresponding number of cells is directly obtained from the graph. Samples were stocked at -80°C on each day and then de-frozen all together at the end of the week.

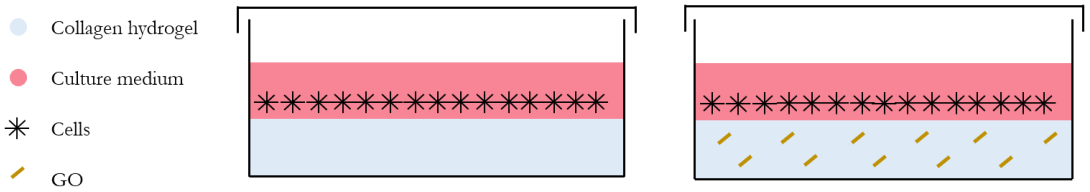


Fig. 3.1 Schematic illustration of two-dimensional cell culture on collagen and GO-collagen hydrogels

3.3.2 Embedded cell culture and contraction assay

New reconstituted collagen solutions, with and without GO, were prepared according to the initially mentioned procedure and cell-seeded with NIH3T3 cells [fig. 3.2] before gelation, as recommended by *Nitta Collagen Inc*® protocol. One collagen hydrogel and three other types of hydrogels containing 0.5%, 2% and 5% exfoliated GO were fabricated. Three samples for each type of hydrogel, including collagen alone, were prepared. Each sample was obtained by seeding 300 µl of reconstituted collagen solution with $5x10^4$ cells before transferring it into a low attachment surface polystyrene 24-well plate. The plate was then placed in a 37°C incubator for 1h to allow for gelation. After that, the gels were carefully detached from the sidewalls of each well by using a sterile scalpel and 400 µl of culture medium were added on top of each gel to make it float. Floatation was required to guarantee a contraction free from any restriction. The plate was then incubated once again at 37°C for 10 days to allow cells for growing. Culture medium was removed every 3 days and substituted with some new media after having thoroughly washed the hydrogels with PBS 1x. Pictures of cells growing inside the gels were taken at day 5 and 10 by using an optic microscope at 10x magnification. The contraction of the hydrogels resulting from the traction forces exerted by the growing cells was evaluated after 10 days, by measuring the diameter with a calibre. The ratio between the final and the initial diameter was calculated to estimate the percentage of contraction.

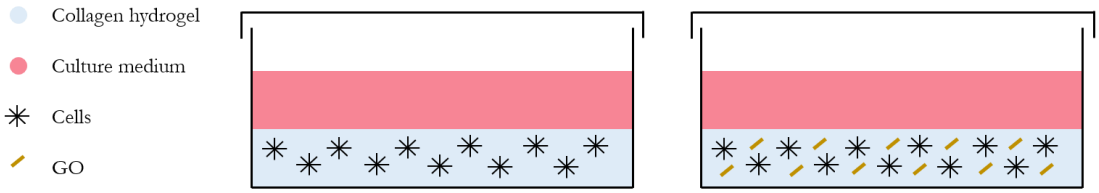


Fig. 3.2 Schematic illustration of three-dimensional cell culture in collagen and GO-collagen hydrogels

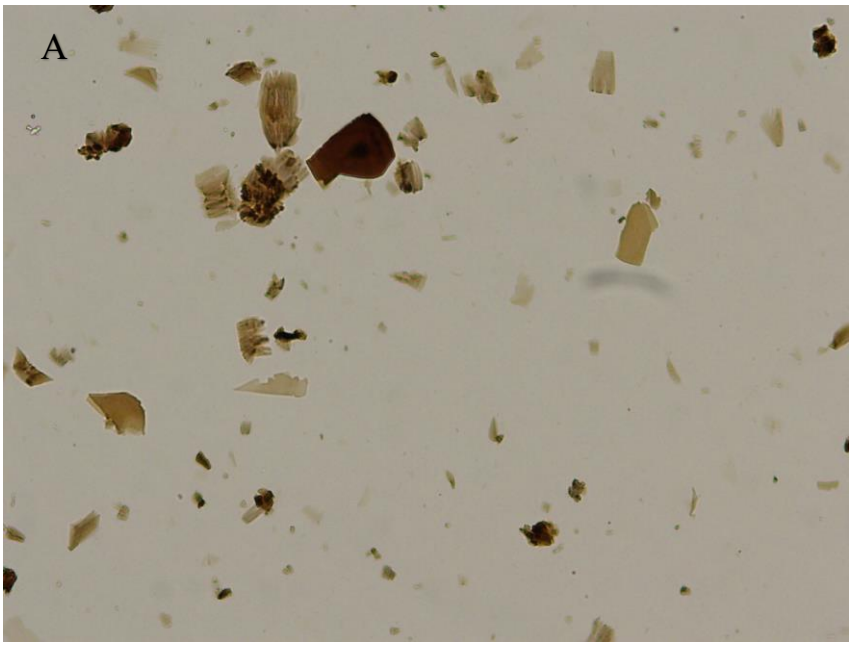
5. Results and discussion

5.1. Preparation of the GO-collagen composite hydrogels

5.1.1. Optical microscope analysis and exfoliation of the GO suspension

A crucial step for the fabrication of a reproducible composite material is confirming that the fillers did not undergo any aggregation before being used as reinforcers. The reinforcement of the matrix of a composite material is, in fact, strongly related not only to the nature of the matrix-filler interaction but also to the extension of the matrix-filler interface inside the material. In fact, given that the interaction is effective, the degree of reinforcement is proportional to the extension of the matrix-filler interface, i.e. to the surface area of the filler.

One of the main advantages of using nanofillers is their highly extended surface area. For a given volume of filler, a wider surface area leads to an enhanced interactions between the filler itself and the matrix, thus to a more effective reinforcing effect. In case aggregation among fillers occurred, the filler-matrix interaction would be diminished due to the establishing of filler-filler interactions. This would result in a less effective reinforcement of the matrix than what was ideally expected for the given concentration of filler. For this reason, the oxide flakes were observed under a microscope before [fig. 5.1a] and after [fig. 5.1b] stirring/sonication in order to optimize the fabrication of our GO-collagen nanocomposite hydrogels. Aggregation between the oxides' flakes, in fact, would inhibit the reinforcing effect of the filler for a given concentration due to the decreased extension of interaction surfaces. As a consequence, making sure that the oxides' flakes did not aggregate before use is a necessary step for the fabrication of a reproducible nanocomposite material. Along with this interaction-related effect, the size homogeneity itself has a direct influence on the mechanical properties of the resulting composite and on its reproducibility.



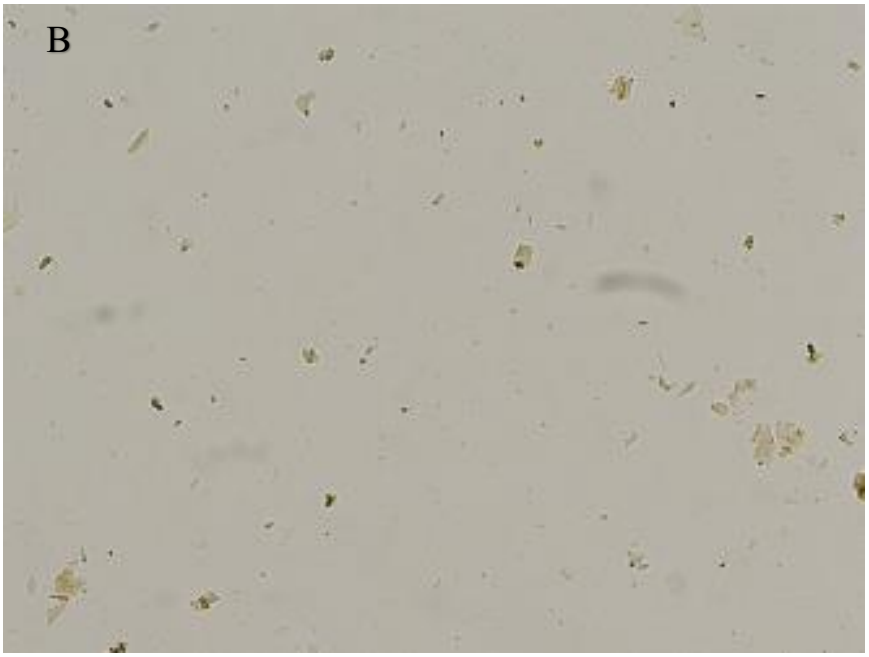


Fig. 5.1 Graphene Oxide (a) before and (b) after undergoing exfoliation via magnetic stirring and sonication

Differences between GO before and after exfoliation were clearly visible. GO from stock aggregated over time giving rise, as a consequence, to an inhomogeneous size distribution of the oxide flakes. Aggregation can be recognized through the presence of darker and bigger flakes in the suspension. After exfoliation, instead, the size distribution was visibly more homogenous and the presence of aggregated flakes was excluded, due to the absence of markedly darker flakes.

The effect of non-exfoliated and exfoliated GO on the properties of our GO-collagen nanocomposite hydrogels will be analysed under many points of view throughout this research work.

5.1.2. Sterilization of the GO suspensions

Multiple sterilization methods were at first considered to treat GO for cell culture applications: UV light, autoclave at 125°C and EtOH 85%. Being the GO suspension dark and opaque, the use of UV light might have led to an unsuccessful sterilization of the oxide. Treatment in autoclave would have instead been responsible for the graphene oxide temperature transition reported to occur at 80°C [45]. For these reasons, sterilization by EtOH 85% was preferred and investigated as a new method.

After the multiple resuspensions in EtOH and DMEM 5x, the concentration of GO in the final suspension turned out to be visibly reduced at eyesight [fig. 5.2]. This was due to the fact that a certain amount of flakes, especially the very fine ones which deposited on the walls of the vessel during centrifugation, got unintentionally sucked away during the removal of the supernatant.

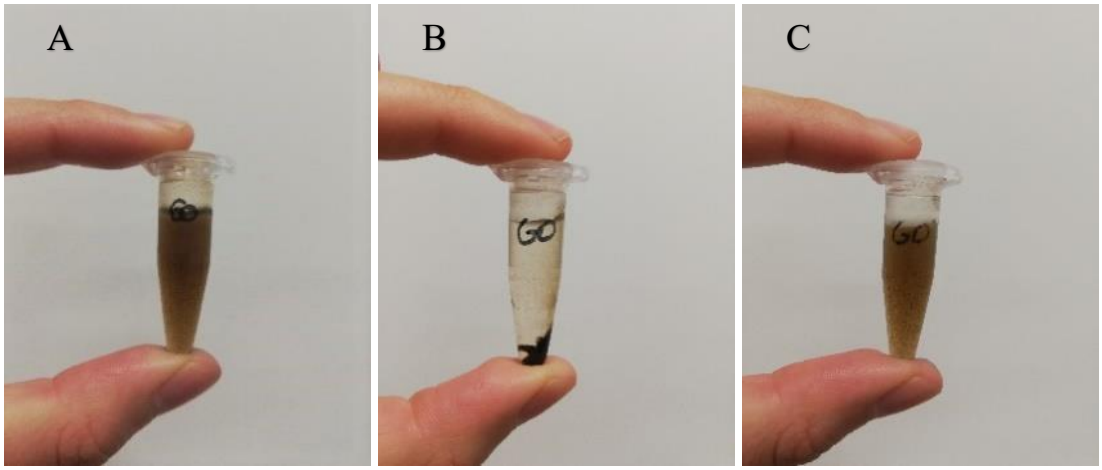


Fig. 5.2 GO suspension (a) before sterilization (b) after centrifugation (c) after final resuspension up to its initial volume. Part of GO was unintentionally sucked away during the multiple removals of the supernatant leading to a final concentration of the oxide lower than the initial one.

Pictures show that the final concentration was visibly lower than the initial one. Being aware of this, the final resuspension in DMEM 5x was performed by adding a significantly lower amount of solution in order to form a suspension of concentration even higher than the initial one. This allowed us to properly dilute the suspension down to the desired initial concentration after proper evaluations through TGA analysis.

5.1.3. Adjustment of the final concentration of the sterilized GO suspension

Thermogravimetric analysis of the GO suspension after its final resuspension in DMEM 5x was performed in order to evaluate the amount of oxide contained in 30µl of suspension, with respect to the amount expected to be found in a 1 mg/ml suspension like the initial one.

TGA of GO suspension [fig. 5.3a and 5.3b] was characterized by a first loss of weight up to around 130°C that can be attributed to the loss of water contained in DMEM 5x. The prolonged evaporation up to 30 degrees above the typical transition temperature was presumably due to kinetics of the transition itself: while water was evaporating, temperature kept increasing at a speed of 10°C/min before it was all gone. A second loss of weight starting at around 180°C and related to the decomposition of carboxyl and hydroxyl functional groups was observed. The pyrolysis of GO started at around 500°C and was over by the end of the measurement. The residual weight, equal to 3.5%, was attributed to the presence of DMEM5x as suspension medium. By referring to its composition and making a brief calculation of the amount of salts contained in 30µl of its solution, we could in fact confirm the hypothesis of salts being responsible for the residual weight. The non-flat and prolonged over 350°C weight loss observed for the intermediate loss of weight [fig. 5.3b] was also attributed to the presence of salts. The weight loss attributed to moisture and water of crystallization in inorganic salts was in fact reported to start at around 200°C [46].

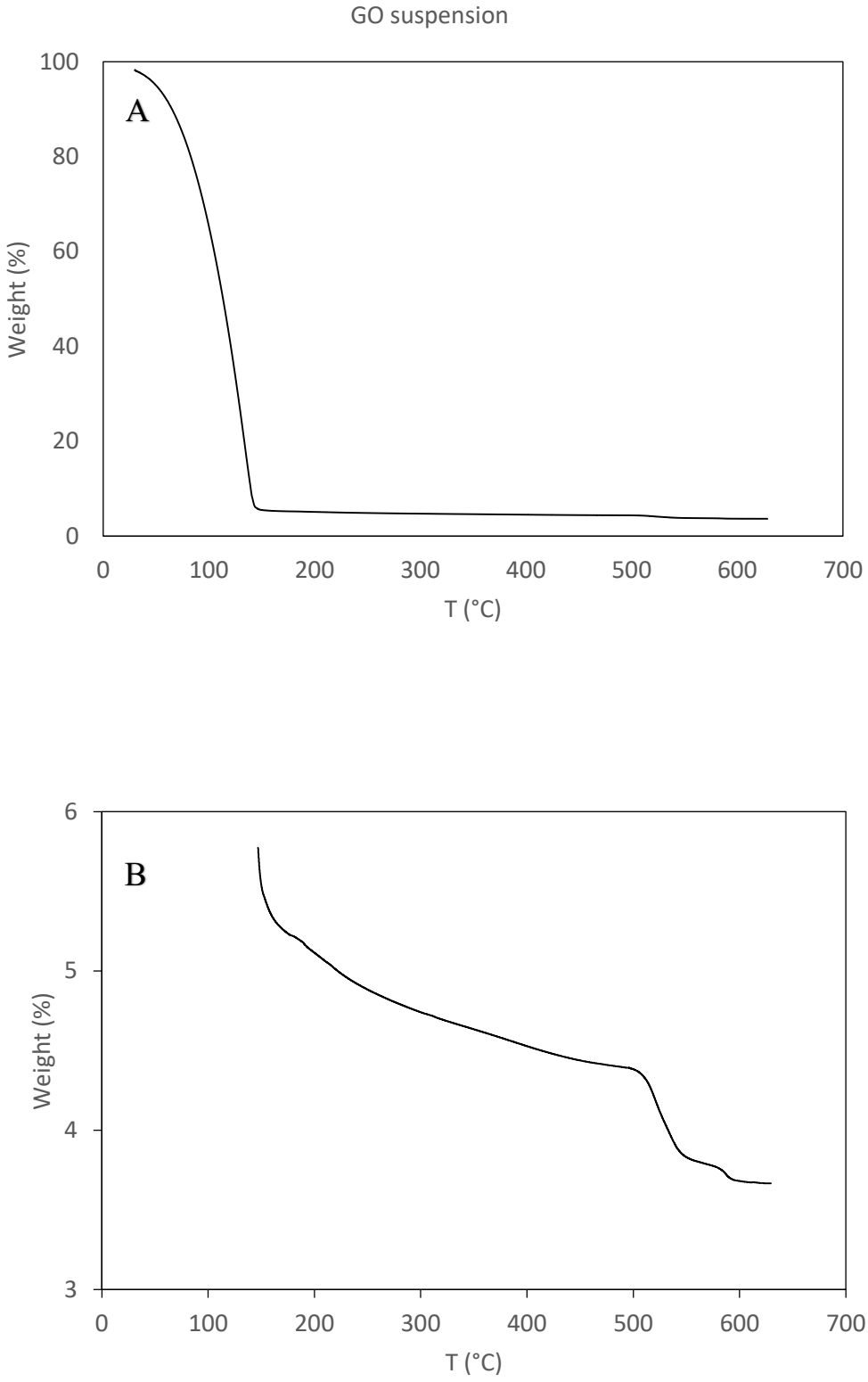


Fig. 5.3 TGA of (a) GO in DMEM 5x (b) GO in DMEM 5x shown on a narrower scale. In the latter, the weight % for the suspension is displayed only up to 6% in order to make the loss of weight related to the pyrolysis of GO more evident than the one due to the high loss of water occurring at the beginning of the measurement.

As a confirmation, TGA of GO powder, obtained from drying at 50°C for 15 hours of the GO-water suspension from stock, was taken as a reference [fig. 5.4]. A similar behaviour to that of the GO suspension was observed up to 130°C, with the only difference that the loss attributed to water was in this case much lower, due to the exclusive presence of moisture in the powder. The intermediate loss of weight was flatter and it was over at around 350°C, which confirmed the above mentioned hypothesis about the presence of DMEM 5x. By comparing graphs of GO powder and GO suspension, it is possible to observe how the former did not show any weight loss at 550°C. This phenomenon is once again attributed to the use of DMEM 5x in case of the GO suspension. The residual weight was down to 1% in case of GO powder.

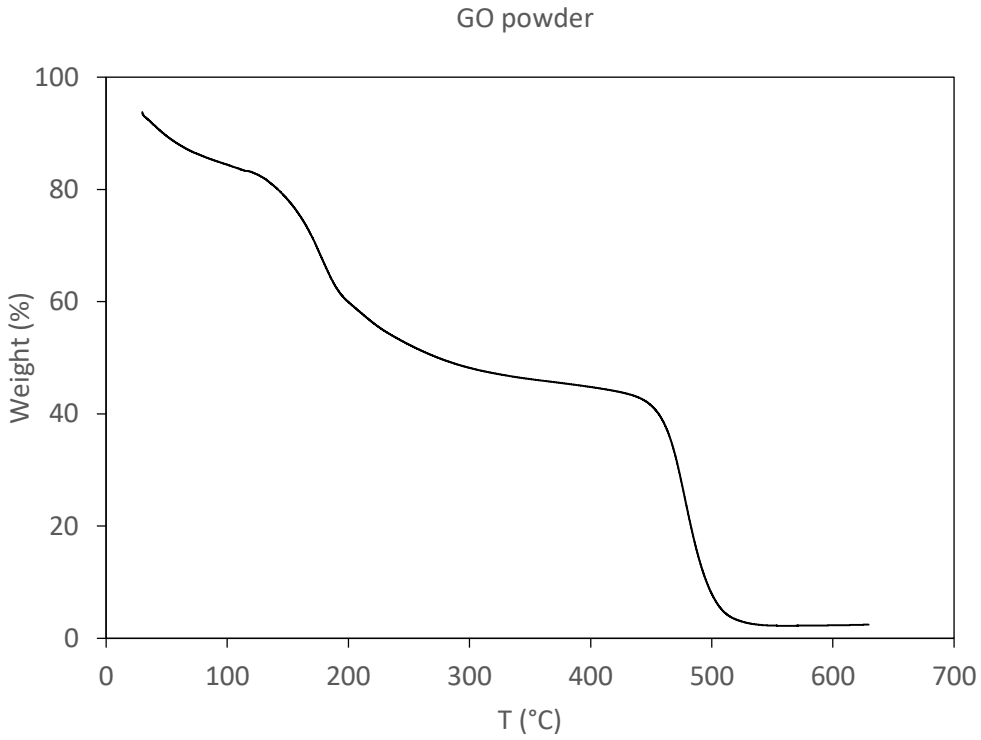


Fig. 5.4 TGA of GO powder analysed under the same experimental conditions as the GO-DMEM 5x suspension

The amount of GO contained in the sterilized suspension was evaluated as the difference between the weight values corresponding to the ending temperature of the measurement (630°C, after the isothermal) and 500°C. TGA analysis of 30 µl of non-sterilized suspension would have led to a GO-related weight loss equal 30 µm. In this case, 36.8 µm of GO were found to be contained in 30 µl of suspension after sterilization, corresponding to a 23% increase in concentration. Being aware of the loss of GO during the multiple removals of the supernatant, in fact, the final resuspension in DMEM 5x was performed up to a lower final volume, it order to obtain a more concentrated suspension that could have been diluted down to the desired concentration after TGA evaluations.

At the end of this experiment, the concentration was adjusted and two GO suspension, 1mg/ml and 0.1mg/ml, were prepared to be used for the synthesis of sterile hydrogels for cell culture.

5.1.4 Synthesis of the hydrogels

The synthesis of our GO-collagen nanocomposite hydrogels according to the previously mentioned procedure was found to be successful. A 0.5% GO-collagen hydrogel is shown below [fig. 5.5] as a representative image for the resulting constructs. Very small GO flakes are visible, and the presence of the oxide is also confirmed by the light-brown color of the hydrogel, which would instead be transparent.

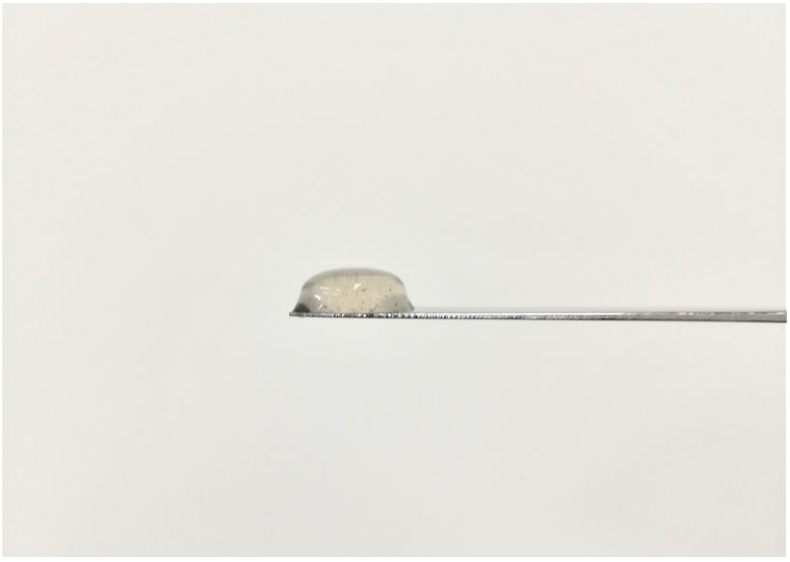


Fig. 5.5 Representative picture of our GO-collagen nanocomposite hydrogels. A concentration of GO equal to 0.5% was used.

Three are the main reasons why we chose to use concentrations of GO in the range between 0,05% and 5%: being GO a small-sized reinforcing filler with a high surface area and aspect ratio, its interaction with the collagen matrix is already maximized for relatively low concentrations up to 5%; collagen is a natural polymer and its self-assembly into helices occurring during gelation might be affected by high amounts of GO; high concentrations of GO might be cytotoxic for cells.

5.2. Characterization of the hydrogels and of the interaction between collagen and GO

5.2.1 Water uptake

For a given hydrophilicity of the macromolecules constituting the three-dimensional network of a hydrogel, the amount of water that can be retained is tightly related to its cross-linking density. In particular, the higher the cross-linking density, the lower the water uptake of the hydrogel. In order for the hydrogel to be able to absorb water, in fact, a certain swelling ability is required. Cross-linking points prevent polymer chains from freely sliding with respect to one

another. This means that the lower their concentration the higher the ability of the hydrogel to absorb water and swell. For this reason, the water uptake decreases as the cross-linking density increases.

The cross-linking density can be then considered a qualitative indicator for the macroscopic mechanical properties of hydrogels [38]. In particular, an increase in network density leads to a corresponding increase in the storage and loss moduli of the hydrogel. In order to increase the density of cross-linking points, the cross-linking process should be controlled. Chemical cross-linking is widely used to enhance the mechanical properties of hydrogels. This method is very effective in terms of final mechanical properties of the hydrogel, being the result of chemical reactions, i.e. of the formation of covalent bonds. Moreover, it allows to control the cross-linking density by varying the amount of cross-linker in solution. Nevertheless, chemically cross-linked hydrogels have the disadvantage of being commonly harmful for cells in culture. In case of physical cross-linking, instead, the risk for cytotoxicity against cells is abolished thanks to the absence of chemical agents inside the hydrogel. However, the final mechanical properties of physically cross-linked hydrogels usually end up being significantly poorer due to the lower amount of energy associated to the breakage of physical bonds with respect to chemical bonds.

Our research focused on the fabrication of GO-collagen nanocomposite hydrogels in which GO acted as a physical cross-linker among collagen fibrils. Collagen and GO physically interact through the hydrogen bonds [fig.5.6] formed between the amine groups of collagen and the hydroxyl groups of GO as reported by Y.Piao et al [35]. Fig. 5.6 shows the interaction occurring between GO and gelatin, the hydrolysed form of collagen. Both chemical and hydrogen bonds are illustrated, being their research focused on the chemical cross-linking of collagen by GO at high temperatures. Only hydrogen bonds are representative of the interaction occurring between collagen and GO in our nanocomposite hydrogels.

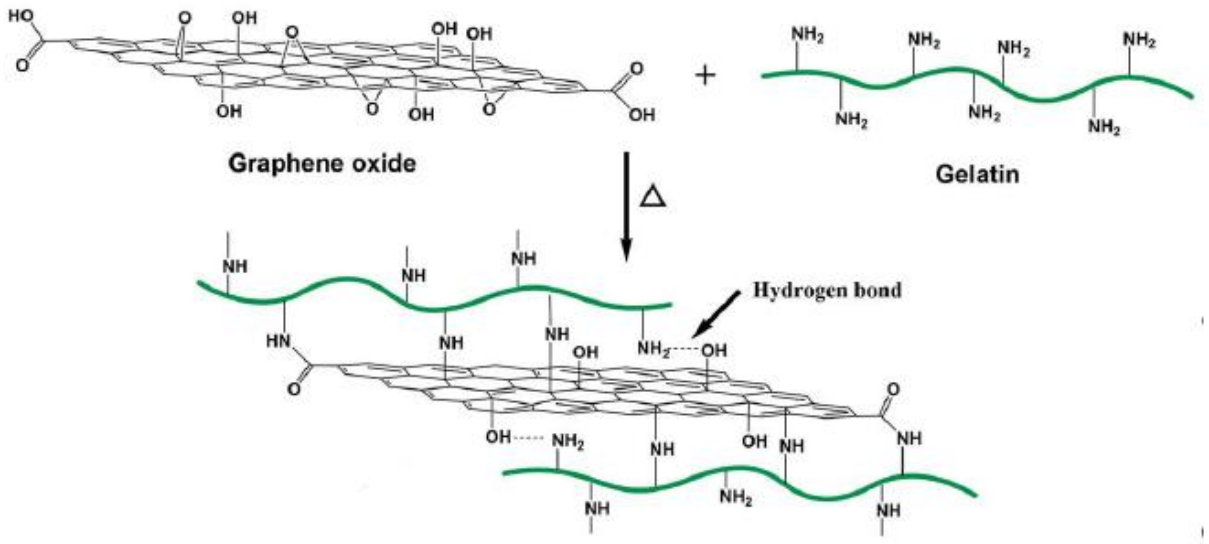


Fig. 5.6 Chemical and physical cross-linking of GO to collagen illustrated by Y. Piao et.al [35]. Only hydrogen bonds are representative of the interaction occurring between collagen and GO in our nanocomposite hydrogels.

By weighing our hydrogels both in wet and dried conditions, the water uptake of our collagen hydrogels charged with both non-exfoliated and exfoliated GO was evaluated. The effectiveness of physical cross-linking is in this case strongly related to the positioning of GO flakes inside the hydrogels. Whether they are all in contact with collagen fibrils or not is hard to establish, being some flakes likely to locate in the water-containing free volume of hydrogels. Those flakes do not contribute to the cross-linking of collagen fibrils.

A similar water content trend was observed for both non-exfoliated [fig. 5.7] and exfoliated [fig. 5.8] GO above 0.5%. The overall effect of the former is a decrease of water content with respect to the reference collagen hydrogel (grey line on all graphs), while the latter led to an overall increase. Hence, an increase and decrease of the cross-linking density of hydrogels were observed respectively. This means that non-exfoliated GO, despite its less homogeneous size distribution, was more effective in terms of cross-linking ability towards collagen. Despite the different behaviours, the effect of both filler on the water uptake was not remarkable. For this reason, two graphs on a narrow scale from 90% to 100% were added in order to make the two trend clearer to understand.

By looking at fig. 5.7a and 5.7b it is possible to observe that the lower water content, i.e. the highest cross-linking density, was obtained for a concentration of non-exfoliated GO equal to 0,5%. According to the displayed results, we can then conclude that non-exfoliated GO is more effective for intermediate concentrations. This might sound counterintuitive, since an increase in the concentration of a filler is usually expected to lead to a more effective reinforcement of the matrix, or to a saturation of the reinforcing effect above a certain threshold concentration. Nevertheless, it should be taken into account that collagen is a natural polymer whose gelation implies a process of self-assembly into fibers that does not allow to automatically extend this reasoning. Moreover, it should be noted that hydrogels possess a free volume filled with water where GO can disperse, reducing its cross-linking effect as if it was inserted at lower concentrations. By neglecting this last phenomenon, our explanation for the overall trend observed in case of collagen hydrogels charged with non-exfoliated GO is that, for very low concentrations of GO up to 0.2%, the amount of graphene oxide flakes was too small to significantly act as a physical cross-linker between collagen fibrils during the process of gelation, even if a slight decrease in the water uptake observed. This decrease was representative of an enhanced interaction, i.e. physical cross-linking. For this reason, by further increasing the concentration of GO the interaction was expected to increase as well. Nevertheless, this was true only up to 0.5% GO, while concentrations above 1% did not turn out to be that effective. We suppose that concentrations above 1% might interfere with the fibrillogenesis of collagen, leading to non-properly self-assembled fibrils and thus, to weaker hydrogels. Despite this, the amount of retained water for GO above 1% is still similar to that of the collagen hydrogel, presumably thanks to an equilibrium between the two opposite effects: the interfering and enhancing one. For this reason, an intermediate concentration of 0.5% turned out to be the most effective content of GO, leading to an optimized physical binding of fibrils.

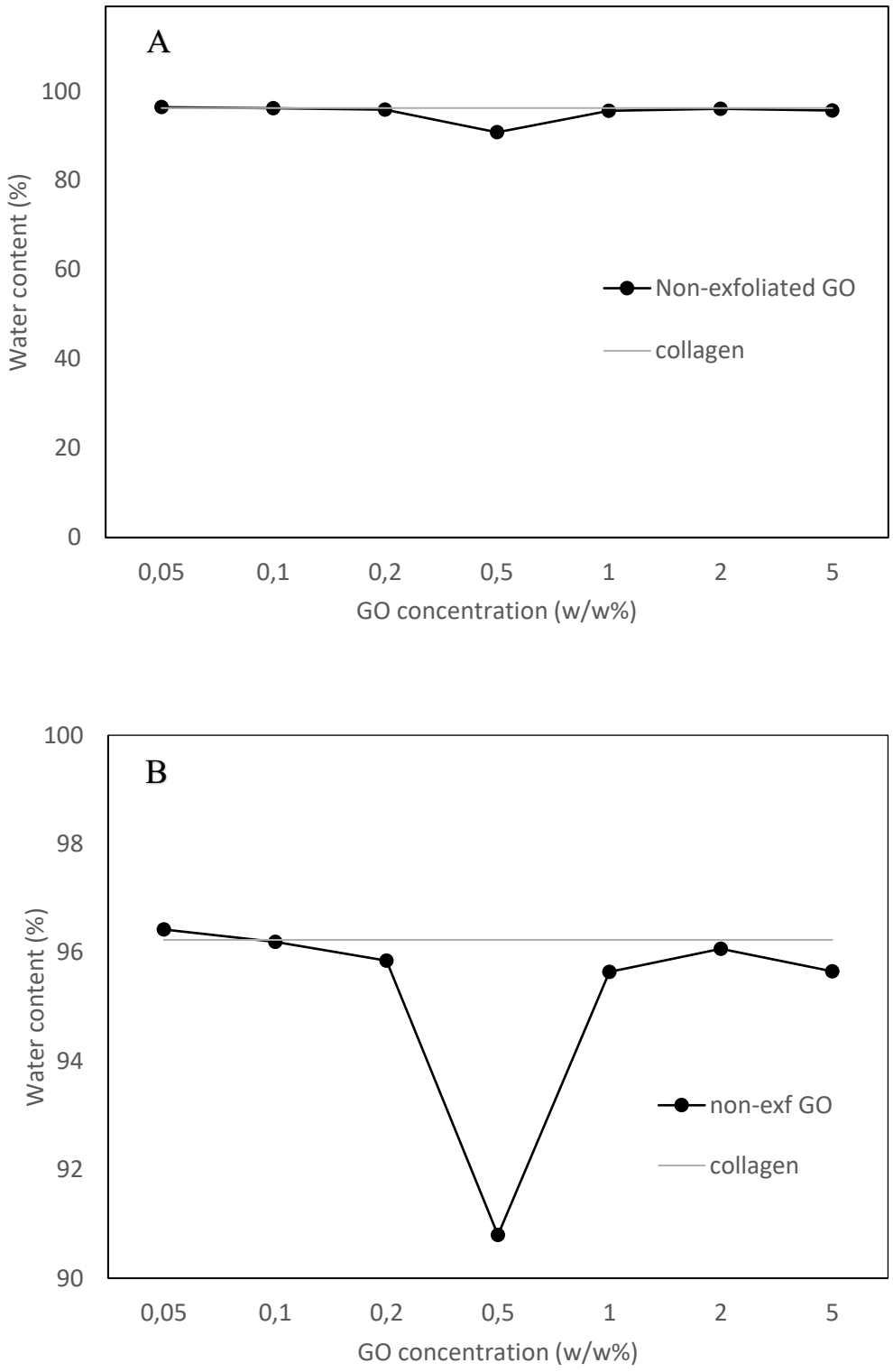


Fig. 5.7 (a) Water content of non-exfoliated GO-collagen hydrogels at increasing concentrations of GO from 0.05 to 5% w/w. (b) The same trend was highlighted on a narrow scale.

In case of exfoliated GO [fig. 5.8a and 5.8b], instead, an increasing water uptake with the increasing concentration of oxide was observed from 0.05 to 5%. The trend above 0.5% was

the same as non-exfoliated GO, even if the amount of water resulted to be higher than that of the reference collagen hydrogel in the whole range of concentrations. This was representative of an overall worsening of the hydrogels' properties.

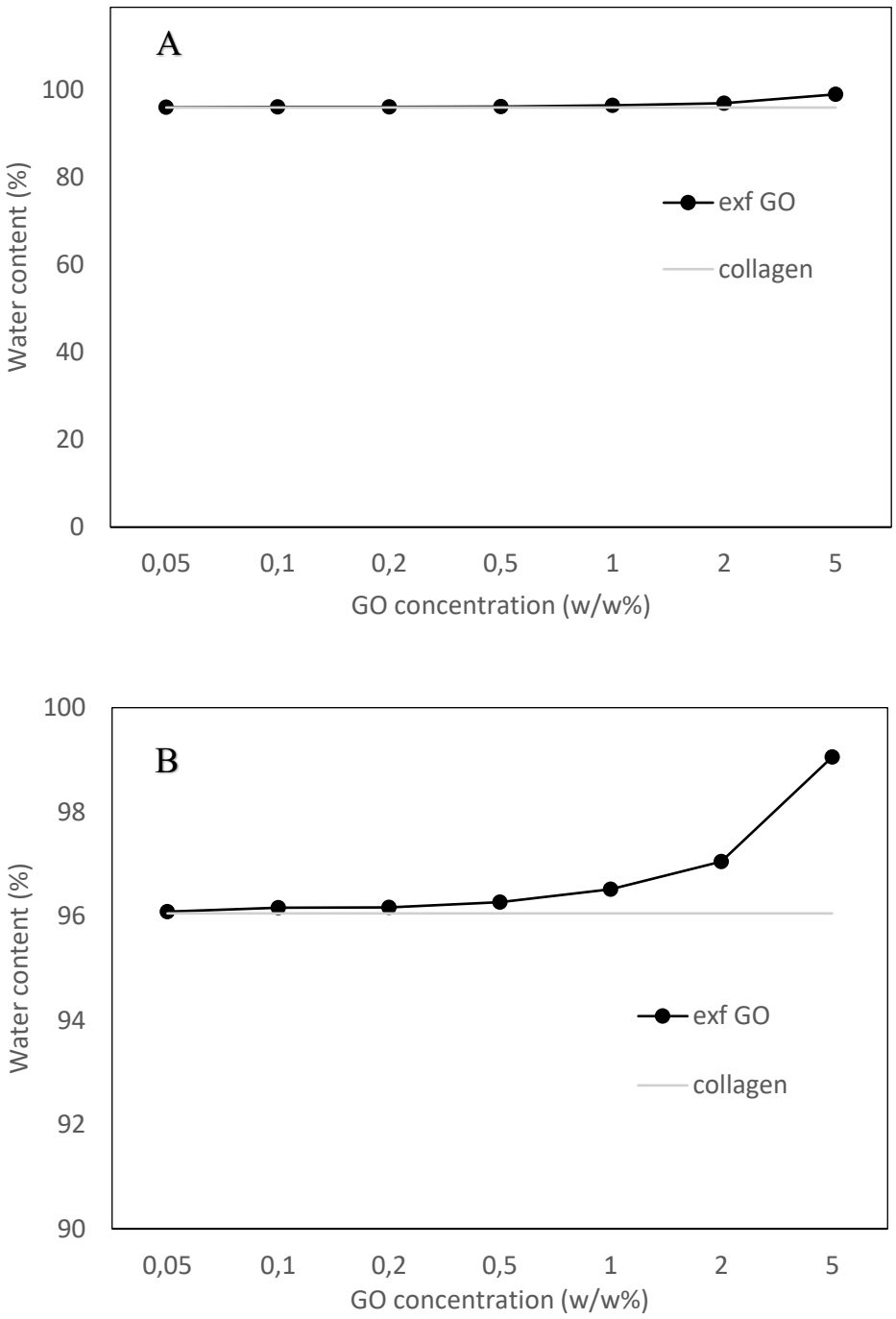


Fig. 5.8 (a) Water content of exfoliated GO-collagen hydrogels at increasing concentrations of GO from 0.05 to 5 w/w%. (b) The same trend was highlighted on a narrow scale.

A more detailed explanation on the reason why the water content of hydrogels charged with exfoliated GO is higher than those charged with non-exfoliated GO and, more importantly, than non-charged collagen hydrogels, will be given through the discussion of the CD results.

5.2.2 CD spectroscopy

Circular dichroism spectroscopy is the most widespread technique used to identify and quantify the secondary structure of proteins and polypeptides in solution [39]. This technique is commonly used to distinguish between ordered (α-helix and β-sheet) and unordered (random coil) structures. CD detects how right-handed circularly polarized light (R-CPL) and left-handed circularly polarized light (L-CPL) is differently absorbed by optically active molecules at different light wavelength. Peptides and proteins, in fact, contain one or more chiral chromophores, which are light-absorbing groups. More specifically, the amine chromophore of the protein bond dominates the CD spectra of proteins below 250 nm [10,12].

The secondary structure is sensitive to its surrounding environment, especially to pH, temperature, and ionic strength, as well as to the presence of small molecules or solutes. As a consequence, a primary use of CD spectroscopy is in detecting the effect of these environmental parameters on the stability and conformation of macromolecules.

The measured signal is defined as ΔA and it is the difference between the absorbance of L-CPL and R-CPL. Starting from this value, the software usually evaluates the ellipticity θ and gives it as the output for the measurements. Between ΔA and θ the correlation showed through eq. 5.1 has been demonstrated to exist:

$$\Delta A = \frac{\theta}{32{,}982} \tag{5.1}$$

Of particular interest is the molar ellipticity [θ], which enables the result to be independent of the concentration of the protein. For our measurements, in fact, an increasing amount of GO ranging from 0.05% to 5% in concentration was added to 300µl of collagen solution 0.3mg/ml to evaluate its effect on the secondary structure of collagen. This led to a gradual decrease of the collagen concentration in the final solution. Being the intensity of the signal also dependent on the concentration of the protein in solution, a normalization of the results was required in order to isolate the effect of the increasing concentration of GO from the that of the decreasing concentration of collagen. The molar ellipticity is defined according to eq. 5.2:

$$[\theta] = \frac{100x\theta}{Cxl} \tag{5.2}$$

where C is the molar concentration of the protein and l the cell pathlength in cm. The factor of 100 converts to pathlength in meters.

Collagen possesses a triple helical structure that gives rise to a peculiar CD spectrum which exhibits two distinct transitions: a positive peak at 222 nm and a negative peak in the range 195-198 nm [40]. The former is representative of collagen triple helices, the latter is associated with polyproline chains [41]. It has been reported that, upon denaturation of collagen, the positive peak disappears while the negative peak is red-shifted and decreased in magnitude. This means,

for example, that a decrease in the intensity of the positive peak is related to a gradual unwinding of the triple helical structure. The absence of this peak is characteristic of random conformation of polypeptide chains [42]. As a consequence, a collagen solution showing such a CD spectrum indicates that the protein has lost its biological activity.

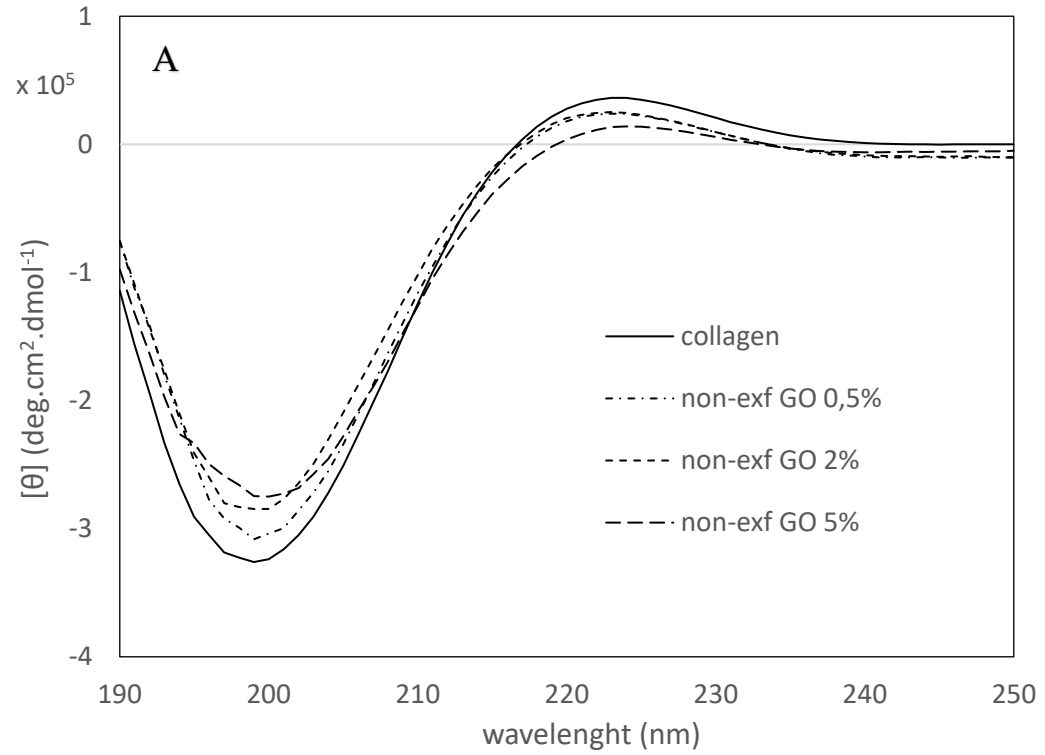
In many cases, both positive and negative peak can be modified as a result of the interaction. For this reason, the most significant parameter which is usually evaluated through the CD spectra is the ratio of the positive and the negative peaks, indicated as Ratio positive-negative (Rpn). The Rpn is a standard indicator for evaluating the efficiency of the triple helical structure of the protein [43], which is an important molecular feature influencing collagen stability, mechanical properties and biofunctionality [16].

In our experimental setup, measurements were carried out at 4°C to impede the fibrillogenesis of collagen. Under acidic conditions, in fact, fibrillogenesis cannot occur, not even at higher temperatures [11]. In our case, though, the collagen solution was neutralized through the addition of buffer and DMEM 5x to reproduce the same condition as that occurring during the gelation of the hydrogels. For this reason, the temperature was kept down to 4°C to prevent collagen from self-organizing during the measurement.

In particular, CD spectroscopy measurements were taken at concentrations of GO equal to 0.5%, 2% and 5% for both exfoliated and non-exfoliated GO right after its addition and after 4h of incubation at 4°C. The influence of GO on the intensity of the positive peak, on the shift of the negative peak and on the Rpn of the spectra was evaluated and compared to the values corresponding to collagen alone. The purpose was to analyse the effect of the concentration and exfoliation of GO and of the incubation time.

For simplicity, only the results after 4h of incubation will be shown. This choice was led by the fact that, in most of cases, the effect of GO immediately after addition was not significantly different from the one observed after 4h. This means that GO has a nearly immediate effect on the secondary structure of collagen. The results for non-exfoliated [fig. 5.9a] and exfoliated [fig. 5.9b] GO are show below. The reference spectrum of collagen is represented through a continuous line in order to make it quickly distinguishable from the other curves. It should be noted, though, that the characteristic peaks of the two reference collagen spectra, for non-exfoliated and exfoliated GO, differ from one another in terms of intensity. This is because at the time of the experiment the amount of collagen solution was not enough to test all the samples. For this reason, a small amount of it was diluted leading to two collagen solutions at different concentrations: 0.3 mg/ml in case of non-exfoliated GO and 0.2 mg/ml in case of exfoliated GO. Nevertheless, the ratio collagen:GO was maintained for both experiments and, as a consequence, the effects of non-exfoliated and exfoliated GO on the secondary structure of collagen was evaluated in relation to their corresponding reference spectrum. Comparisons between the two cases can still be made by analysing the changes observed for each spectrum and confronting the resulting values of Rpn.

The positive peak at 222 nm can be identified for all the spectra in both cases. This means that even after the addition of GO the triple helical structure of collagen was preserved, thus, collagen has maintained its biological activity. This is a fundamental requirement for the protein to be used for cell culture purposes. Despite being preserved, it is not possible to precisely establish at first sight whether the amount of triple helices is still the same or not. In most of cases, in fact, the positive peak showed a slight decrease in magnitude even if a more evident effect was observed for the negative peak at 198 nm related to polyproline. This means that the relative amount of helices has varied.



a)

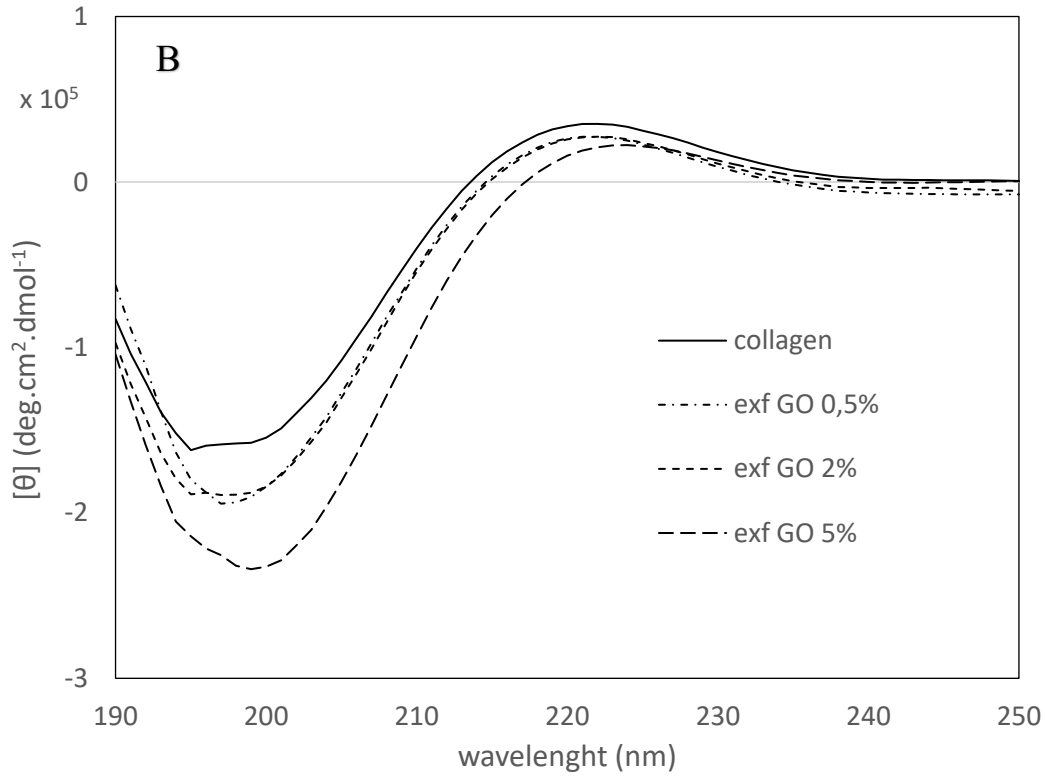


Fig. 5.9 Effect of (a) non-exfoliated GO and (b) exfoliated GO at increasing concentrations on the CD spectra of collagen

By calculating the Rpn for each curve, it is possible to evaluate the exact percentage of triple helices left in the collagen structure after its interaction with non-exfoliated and exfoliated GO. The Rpn was evaluated by considering the molar ellipticity at 222 nm for the positive peak and at 198 nm for the negative peak. The Rpn of collagen 0.3 mg/ml alone was estimated to be equal to 0.11 and then used as a reference for non-exfoliated GO [fig. 7a]. The Rpn values after addition of non-exfoliated GO 0.5%, 2% and 5% were instead equal to 0.108, 0.105 and 0.103 respectively. Normalization of these values with respect to the value of collagen resulted in triple helix contents of 98%, 95% and 93%. These results are not to be intended as extremely precise but on the whole they indicate that the triple helix architecture was preserved, even if its content diminished with the increasing concentration of GO. In case of collagen 0.2 mg/ml, instead, the Rpn was estimated to be equal to 0.21, which decreased down to 0.14, 0.13 and 0.11 after the addition of exfoliated GO 0.5%, 2% and 5% respectively. Normalization of these values led to much lower amounts of triple helices left intact after the interaction with GO: 67%, 62% and 52%. On the whole, this means that exfoliated GO has a destabilizing effect on the secondary structure of collagen.

An explanation for this behaviour can be found in a previous study carried out by Nishina et.al [37] on the interaction between GO and siRNA. GO of two different sizes was used and it was found that small GO tends to intercalate between the double strands of siRNA inducing sever damages on its conformation, while large GO interacts intermolecularly leading to a reduction of the siRNA winding angle without breaking its structure. Collagen has a helical structure similar to that of RNA, even if triple instead of double, and both helices are stabilized by H-bonds between the strands. As a consequence, the same reasoning valid for siRNA might be applied to our study. As a consequence, we supposed the destabilizing effect of exfoliated GO on collagen to be strictly related to its size, being small enough to insert within the collagen strands.

Even if it is not possible to state for sure whether there is a direct correlation between the effect of GO on the triple helices of collagen and the cross-linking density of the corresponding hydrogels, an explanation for the reason why the water uptake of exfoliated GO-collagen hydrogels was higher than the values resulting from the use of non-exfoliated GO can be now given. The Rpn values in presence of exfoliated GO were significantly decreased with respect to collagen and this was representative of a partial denaturation of the collagen helices. The corresponding exfoliated GO-charged hydrogels showed a higher amount of retained water, i.e. a lower cross-linking density than the reference collagen hydrogel. As a consequence, we suppose that the weakening of the collagen structure due to the breakage of the helices is responsible for the formation of less compact networks, in which the density of the physical cross-linking points is further lowered by the fact that most of GO flakes intercalated within the strands instead of enhancing the interfibrillar H-bonds. The combination of these two effects might explain why the cross-linking density decreased by increasing the amount of exfoliated GO. For concentrations below 0.5%, instead, the amount of GO is presumably too low to have any significant effect on the structure of collagen. Moving to non-exfoliated GO, the Rpn values in its presence were all very close to that of the reference collagen solution. This meant that the triple helices were well preserved. The corresponding non-exfoliated GO-charged hydrogels showed a lower amount of retained water, i.e. a higher cross-linking density, than the reference collagen hydrogel. According to what has been said so far, the secondary structure of collagen was not damaged by the presence of GO, which located among fibers enhancing their interaction. This is also confirmed by the decrease in magnitude of the negative peak of collagen after the addition of non-exfoliated GO. Such a decrease has been reported to be representative of a promoted aggregation of collagen [10]. As a consequence, we would expect the cross-linking density to increase with the concentration of GO. Nevertheless, above 0.5% of GO an opposite

behaviour was observed: even if GO did not interfere with the triple helices, it might have interfered with the self-organization of fibrils into fibers (fibrillogenesis) during gelation, leading to the formation of a less compact network. In our experimental conditions, in fact, collagen was kept at 4°C to prevent its fibrillogenesis, so the effect of GO on the organization into fibers was not detected. GO 0.5% is then the concentration leading to an optimal balance between these two opposite effects.

5.2.3 Rheology

The behaviour of collagen hydrogels is determined by the interaction between and the intrinsic properties of the network of collagen fibrils and the interstitial solution [47]. These two phases allow collagen hydrogels to withstand both shear and compressive loads: the entangled network is able to resists shear and extension, while the interstitial solution can flow through the network leading to high pressures that allow the gel to support compressive loads and prevent collapse.

Collagen hydrogels have been reported to be non-linearly elastic and to exhibit strain stiffening, which means that their elasticity increases with the increasing applied strain [44]. Strain stiffening, though, is diminished over time due to strain enhanced stress-relaxation. This phenomenon is the relaxation of a stress as a response to an applied strain: reorganization of fibrils inside the hydrogel allows the relaxation of stresses in the matrix leading to a decrease over time of the initial stress resisting the applied strain. In collagen hydrogels, this behaviour has been demonstrated to be related to the unbinding of fibers due to the force-dependent breakage of weak interactions such as hydrophobic and electrostatic forces [44]. At long timescales, stress relaxation has been found to be caused by lengthening of fibrils instead of their unbinding. Fibrils, in fact, slide with respect to one another until intrafibrillar lengthening occurs. Strain-enhanced stress-relaxation was still observed in case of covalently cross-linked hydrogels, even if to a lower extent.

Because collagen hydrogels consolidate under the traction forces exerted by cells in culture, their mechanical characterization is essential to understand their behaviour when used as tissue equivalents.

Cells on 2D substrates and in 3D collagen hydrogels can exert stresses on the order of 100-1000 Pa and strains of up to 0.5 [44], over a range of frequencies from 0.1 to 10 Hz (approximately from 0.6 to 60 rad/sec) that well represents the stress that collagen scaffolds may experience in vivo during tissue remodelling processes [47]. Our hydrogels were tested in strain controlled conditions over an angular frequency range from 0.1 to 100 rad/s under a fixed strain of 5%. Results for non-exfoliated [fig. 5.10] and exfoliated [fig. 5.11] GO-collagen hydrogels are shown below. Both G' (storage modulus) and G'' (loss modulus) are displayed, in order to highlight the effect of GO on the stiffness and on the stability of hydrogels' networks.

In case of hydrogels charged with non-exfoliated GO, both moduli G' and G'' increased with ω, reflecting the typical relaxation mechanism of collagen hydrogels. G' was significantly higher than G'', indicating that hydrogels had a substantial elastic response and that their network was stable and continuous. Furthermore, G' and G'' had the same trend and were substantially parallel, except for 5% GO, leading to a stable loss tangent δ (G''/G'') for nearly all hydrogels.

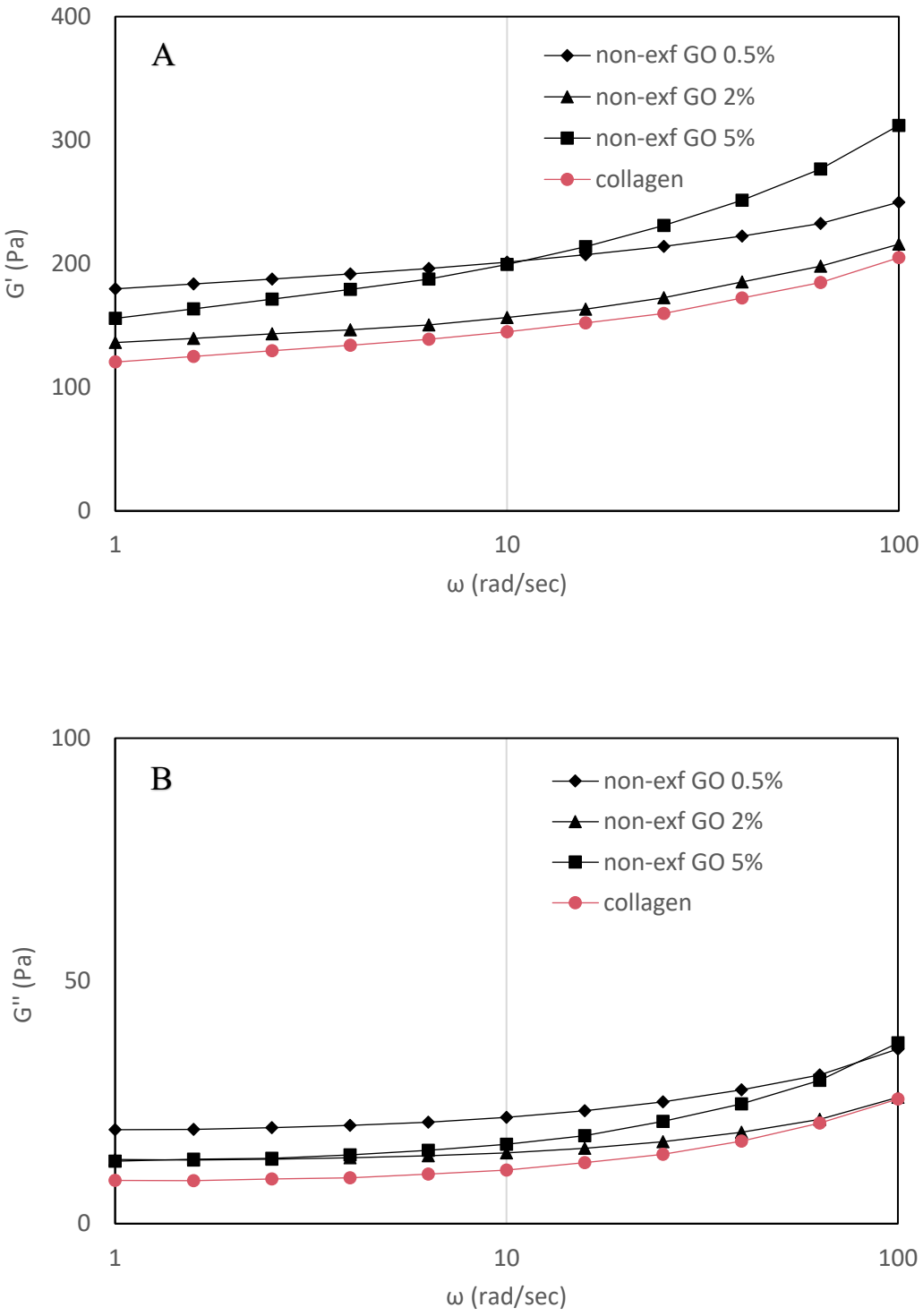
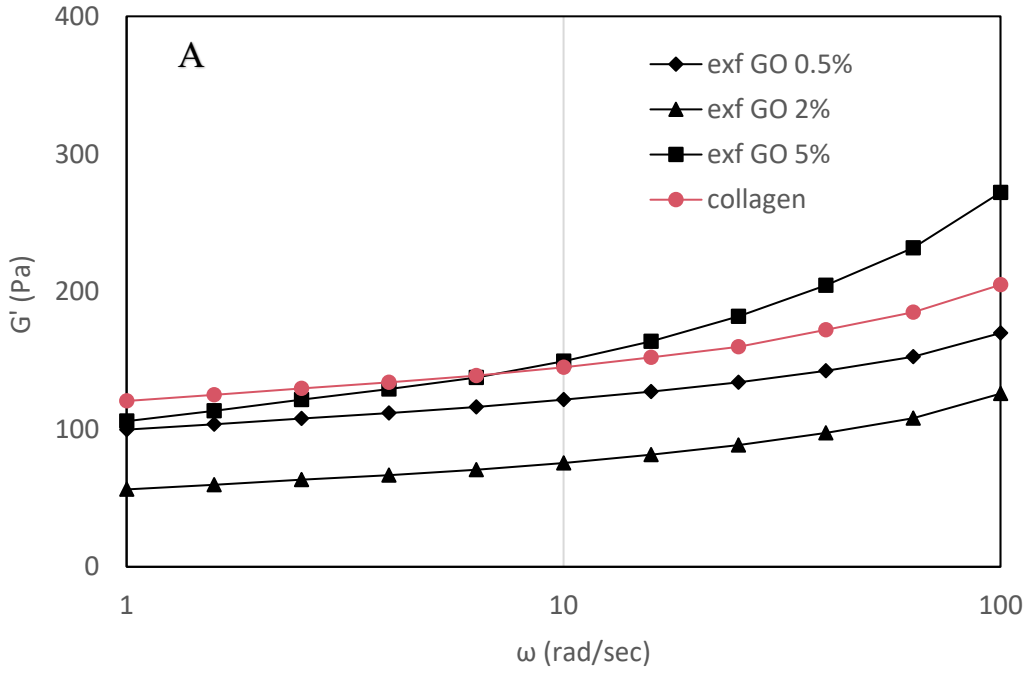


Fig. 5.10 Viscoelastic response of collagen and non-exfoliated GO-collagen hydrogels. The frequency dependence of both (a) storage modulus G' and (b) loss modulus G'' was tested in oscillatory shear at a constant strain amplitude of 5%.

According to the graphs, the rheological properties of the hydrogels depend on the concentration of GO. In particular, 0.5% GO led to a 50% increase in the value of G' (180 Pa)

with respect to the reference collagen hydrogel (120 Pa). 2% GO resulted only in a small improvement limited to 135 Pa, corresponding to a 12,5% increase. At this point, 5% GO was expected to lead to an improvement even less significant than that resulting form 2% GO, also according to the previous water and CD spectra evaluations. Nevertheless, a 29% increase was observed. A presumably valid explanation for this behaviour is the double effect that GO has on collagen hydrogels: on one side the fibrillogenesis of collagen is affected by the increasing concentration of GO which leads to less compact hydrogels; on the other side GO is responsible for a stiffening given by its intrinsically high mechanical strength. At high concentrations of GO such as 5%, the influence of the intrinsic strength of GO prevails on the inhibiting effect that it has on fibrillogenesis. The effect of the high concentration of GO can also be seen by the fact that both moduli of the 5% GO-collagen hydrogel increased with ω in a more significant manner than those of the other hydrogels. As a consequence, the reciprocal time constant at 100 rad/sec is significantly increased, meaning that the time required for the relaxation to occur is longer. This is reasonable if we consider that high concentrations of GO might interfere with the relaxation mechanisms occurring in the fibrillar network.

In case of exfoliated GO-collagen hydrogels, the relaxation mechanism was still visible and G' was still higher than G'' even if the overall values of δ were lower when compared to the previous case, meaning that exfoliated GO led to the formation of less stable networks. Moreover, values of G' of the exfoliated-GO charged hydrogels were lower than that of the reference collagen hydrogel, meaning that the addition of exfoliated GO led to a worsening of the hydrogels' stiffness. These results are in accordance with the previous experimental outcomes. By comparing exfoliated GO with non-exfoliated GO-collagen hydrogels, it is possible to notice the same overall GO-dependent behaviour. Even in presence of exfoliated GO, in fact, a decrease of G' was observed with the increasing concentration of GO, except for 5% GO. in particular, 0.5% and 2% GO led to a 15% and 54% decrease of G' respectively, while 5% GO led to a 25% increase.



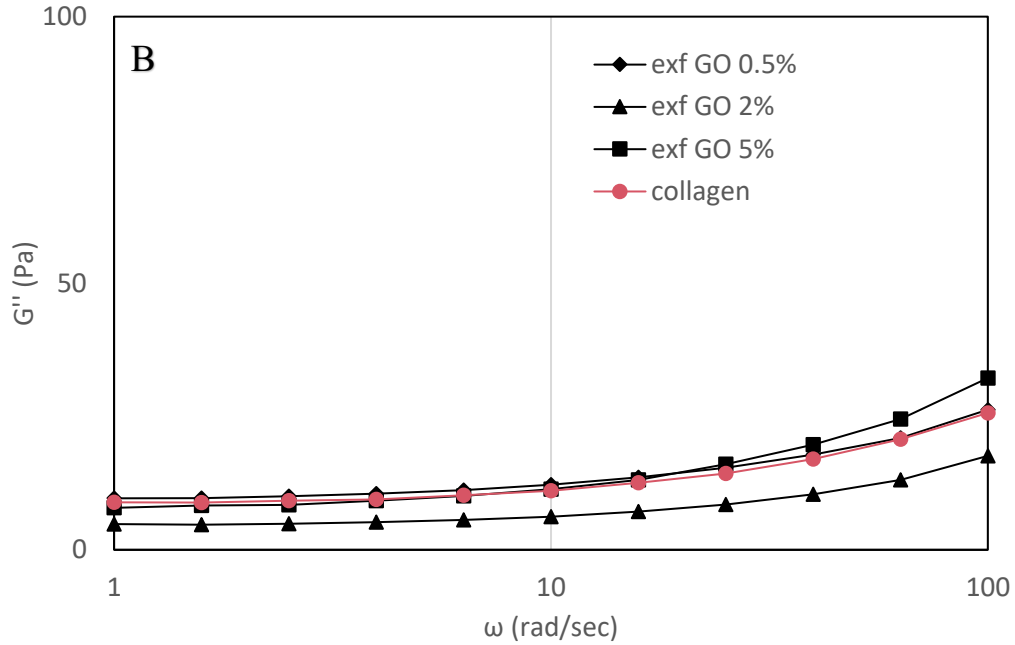


Fig. 5.11 Viscoelastic response of collagen and exfoliated GO-collagen hydrogels. The frequency dependence of both (a) storage modulus G' and (b) loss modulus G'' was tested in oscillatory shear at a constant strain amplitude of 5%.

G' and G'' have been reported to increase with the increasing cross-linking density and, to a higher extent, with the increasing concentration of collagen [47]. As a consequence, being the concentration of collagen constant in all of our hydrogels, the trend observed for G' and G'' are representative for their GO-dependent cross-linking densities and confirm the water content results, except for 5% GO for the previously mentioned reasons. We can in fact conclude that GO at high concentrations leads to the formation of hydrogels whose mechanical behaviour is mainly determined by its own intrinsic properties.

5.2.4 SEM

Dehydration of the samples for SEM analysis via multiple immersions in EtOH at increasing concentrations might have led to a release of the GO loaded in the hydrogels. Nevertheless, no traces of GO were observed in the solvents after the immersions, presumably thanks to the fixation previously carried out on the hydrogels with glutaraldehyde.

Fixation is a method widely used in the field of anatomy to prevent biological tissues from decay and to preserve their morphology for further analysis. In case of chemical fixation, chemical fixatives such as glutaraldehyde are used. Glutaraldehyde acts as a cross-liker by chemically reacting with the functional groups found in proteins.

Fixatives enable to keep the structure of tissues as close to their original form as possible. Nevertheless, it is important to remember that even the most careful fixation leads to a morphologic alteration. When fixation is performed via immersion in the fixative agent, such as in our case, the agent must diffuse through the sample to fix it. This means that the sample's size is crucial for the final result. For this reason, particular attention to the diameter and thickness of our hydrogels was payed during their preparation, in order to be able to make a valid comparison among the samples' morphology. Given this, some visible differences among

the cross-linking densities are expected to be found, according to the results previously obtained through the water uptake and CD spectroscopy.

Fig.8b/c and fig.9b/c show the morphology of 0.5% and 5% GO-collagen hydrogels under SEM in case of non-exfoliated and exfoliated GO respectively, compared to the same collagen hydrogel [fig. 5.12a and 5.13a]. Collagen fibers and their entanglements are clearly visible in all images, which highlight the formation of an open and interconnected porous structure. It is possible to notice an overall effect of GO, more or less significant depending on the case, on the cross-linking density of the hydrogels. If we first consider collagen hydrogels charged with non-exfoliated GO [fig. 5.12], we can notice how the presence of the oxide [fig. 5.12b/c] led to the formation of a more dense network during gelation with respect to the non-charged collagen hydrogel [fig. 5.12a]. In particular, the most significant effect is given by a concentration of non-exfoliated GO equal to 0.5% [fig. 5.12b], even if there is no remarkable difference when compared to 5% GO [fig. 5.12c]. Being SEM imaging only a qualitative evaluation of the cross-linking density, in fact, it does not allow to draw detailed conclusions. Nevertheless, the result is an accordance with the outcomes previously observed through the water uptake and CD evaluations. In fact, the lowest water uptake (91%), which is related to the highest cross-linking density, was found to be corresponding to a concentration of oxide equal to 0.5%. Similarly, the hydrogel containing 5% non-exfoliated GO showed a slightly lower water uptake, i.e. a slightly higher cross-linking density, with respect to the collagen hydrogel, but not as relevant as 0.5% GO. The same trend can be observed in the SEM images: the collagen hydrogel shows the lowest cross-linking density, which increases when GO is added, in particular when it is introduced at a concentration of 0.5%.

On the other hand, if we consider collagen hydrogels charged with exfoliated GO [fig. 5.12], we can see how its effectiveness on the resulting cross-linking density is reduced if compared to non-exfoliated GO, regardless of its concentration. This is in accordance with the hypothesis that smaller GO intercalates in the triple helixes of collagen giving rise to a less compact network, also because most of GO in this case does not contribute to the formation of the physical cross-linking points. In particular, by looking at fig. 9b and 9c it is possible to notice how both 0.5% and 5% exfoliated GO did not lead to a higher cross-linking density than before. Once again, this result confirms the previous findings, which showed that the water uptake of collagen hydrogels charged with exfoliated GO was higher than that of hydrogels charged with non-exfoliated GO. Hence, their cross-linking density was supposed to be lower.

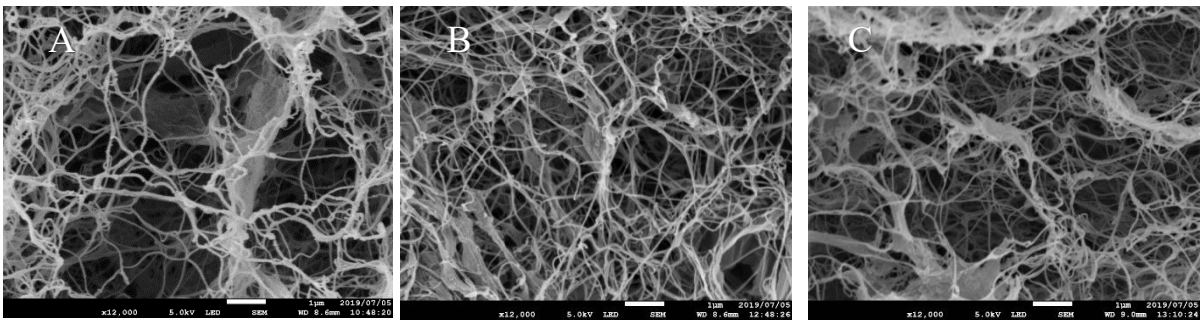


Fig. 5.12 SEM images of (a) collagen (b) 0.5% GO-collagen and (c) 5% GO-collagen hydrogels. GO is non-exfoliated.

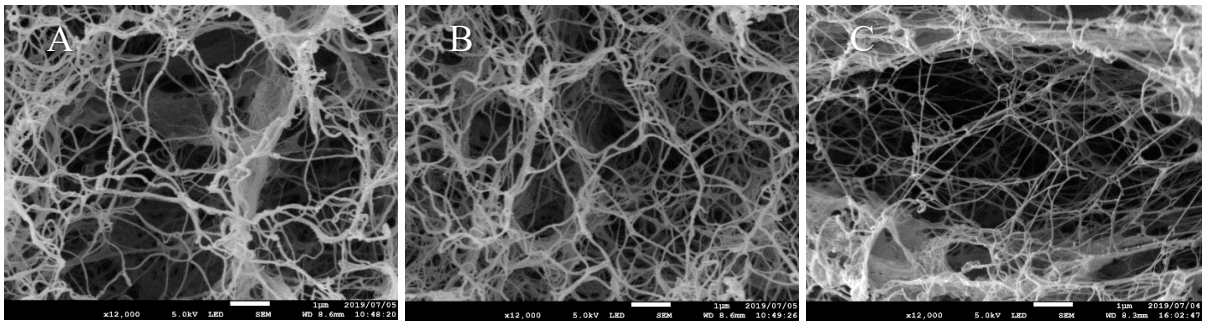


Fig. 5.13 SEM images of (a) collagen (b) 0.5% GO-collagen and (c) 5%GO-collagen hydrogels. GO exfoliated.

On the whole, despite being only a qualitative analysis of the hydrogels morphology and not a quantitative evaluation of the cross-linking densities, SEM images confirmed the hypothesis that were previously put forward through water uptake evaluations. According to rheologic measurements, the increase of cross-linking density observed in case of 0.5% is real even if not significant. Because GO acts as a physical cross-linker, in fact, the enhancement of the stiffness of our hydrogels is not remarkable if compared to the values of G' found in literature in case of GO-collagen chemically cross-linked hydrogels.

5.3. Cell culture

As partly anticipated, monolayer and embedded cell culture were performed to evaluate the cytotoxicity of GO, the successfulness or failure of its sterilization and the influence of different GO-collagen hydrogels on cell-growth speed and on hydrogels' contraction.

NIH3T3 cells were used. 3T3 is a cell line that has been established in 1962 from mouse embryonic tissues and that has become the standard fibroblast cell line [IV].

5.3.1 2D cell culture

Monolayer cell culture can be used to qualitatively compare the stiffness of different substrates. As initially explained, in fact, cell growth is strongly affected by the mechanical properties of the ECM. Despite not being able to mimic the three-dimensional environment of biological tissues, 2D cell culture is still influenced by the stiffness of the substrate, even if to a lower extent than embedded cell culture. In particular, by counting cells through the fluorescence assay explained in the methods paragraph, it is possible to indirectly get information on the relative stiffness of multiple hydrogels compared to one another. 2D cell culture is more suitable for this purpose, thanks to the possibility of detaching cells more easily. In case of embedded cell culture, in fact, hydrogels should be intensely smashed to allow the extraction of cells, with the risk of not being able to take all of them out, leading to a final interpretation of data which would be far from reality. The fluorescence assay was carried out in order to get a qualitative trend of the hydrogels' stiffness based on the number of cells grown on each hydrogel at day 1, 3, 5, and 7. Unfortunately, the experiment failed due to unknown reasons, probably related to a non-proper detachment of cells from the hydrogels. As a consequence, only images captured under the optic microscope [fig. 5.14, 5.15, 5.16, 5.17]

could be used as a general reference for cell growth. Growth on GO-collagen hydrogels on day 1, 3, 5, and 7 was compared to that on simple collage-hydrogels. Pictures of the more populated area of each hydrogel were taken.

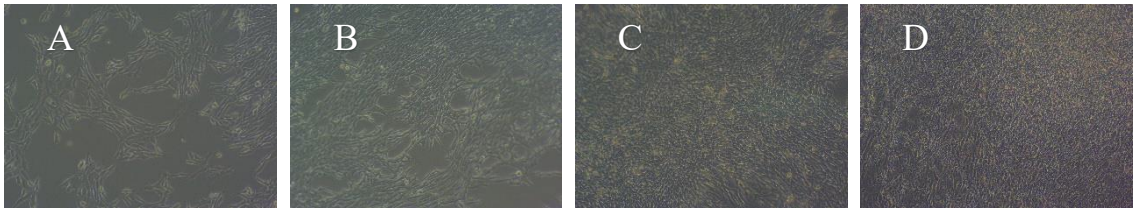


Fig. 5.14 Growth of NIH3T3 cells on collagen hydrogels on day (a) 1, (b) 3, (c) 5 and (d) 7

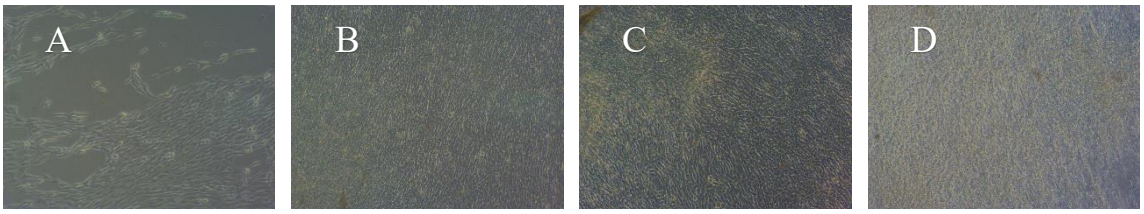


Fig. 5.15 Growth of NIH3T3 cells on 0.5% GO-collagen hydrogels on day (a) 1, (b) 3, (c) 5 and (d) 7

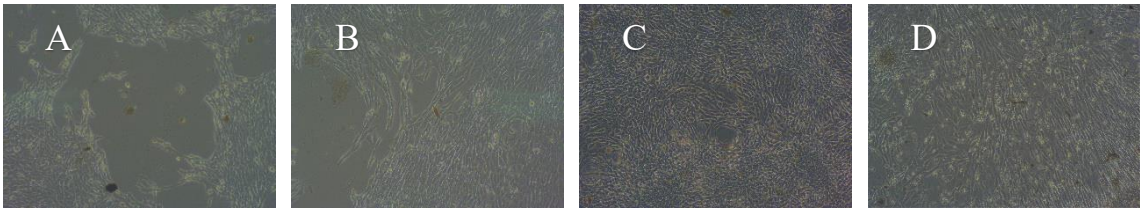


Fig. 5.16 Growth of NIH3T3 cells on 2% GO-collagen hydrogels on day (a) 1, (b) 3, (c) 5 and (d) 7

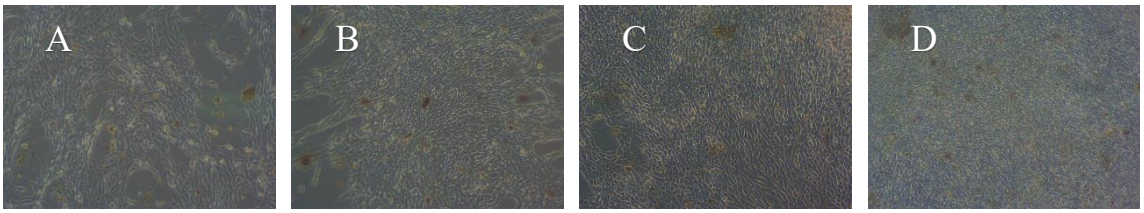


Fig. 5.17 Growth of NIH3T3 cells on 5% GO-collagen hydrogels on day (a) 1, (b) 3, (c) 5 and (d) 7

Each sequence of images clearly shows the progressive growth of cells from day 1 to 7. Differences among the four types of hydrogels were clearly visible on days 1 and 3 while comparisons on days 5 and 7 were more difficult to make at eyesight due to the exponential growth of cells which brought to confluency.

From day 1 to 3, cells showed a similar growth on collagen hydrogel and 5% GO-collagen hydrogel, a slower growth on 2% GO-collagen hydrogel and a remarkably faster growth on

0.5% GO-collagen hydrogel. The same trend was noticed for days 5 and 7. Previous water uptake results showed a decreasing cross-linking density of the hydrogels with the increasing concentration of GO above 0.5%, and the same trend of G' was obtained from rheologic measurements (except for 5% GO due to the intrinsic mechanical strength of the flakes). According to this, the number of cells observed on a certain day should have decreased with the increasing concentration of GO in the hydrogels. Nevertheless, 0.5% GO, 2% GO and 5% GO brought to a significantly faster, similar and slower growth than the collagen hydrogel respectively. To explain this behaviour, we should take in to account that also GO has an influence on cell growth. As reported in the introductory chapter, GO enhances cell attachment and, as a consequence, cell growth. For this reason, the higher the concentration of GO the faster cell growth would be expected to get. Overall, the observed cell growth can be considered as a result of two opposite driving forces: the stiffness of the hydrogels, which decreases with GO, and GO itself. The former prevailing at low concentrations, the latter at high concentrations. This explains the reason why 0.5% GO showed the fastest cell growth, 2% the slowest (even slower than simple collagen) and 5% a growth speed comparable to collagen (balance between the effects of stiffness and GO was reached).

Thanks to the successful growth of cells, we could confirm that the culture environment was sterile. Hence, that the sterilization of GO by EtOH 85% was successful as well. However, no conclusions could be drawn on the cytotoxicity of GO against fibroblasts due to the absence of a three-dimensional interaction between the two in the case of monolayer cell culture. Cytotoxicity of GO, in fact, is determined by its ability to enter cytoplasm and nucleus [33].

5.3.2 Embedded cell culture and contraction assay

Embedded cell culture enables to closely mimic the ECM of biological tissues, both in terms of structure and cell-matrix interaction. When a cell-seeded hydrogel is let free to float, it tends to contract as a consequence of the traction forces exerted by the growing cells. In the particular case of a collagen hydrogel, forces are exerted on collagen network fibers [46]. Cell contractile forces leading to compaction and stiffening are due to the activation of myosine, and contraction proceeds only when traction forces exceed the mechanical resistance of the matrix [46]. In absence of mechanical constraints applied on the hydrogel's boundaries, cells entrapped in the gel assume a stress fiber-free morphology. By evaluating the shrinkage of our GO-collagen hydrogels after 10 days of incubation at 37°C [fig. 5.18] it was then possible to have a qualitative understanding of the GO-dependent stiffness of the hydrogels. The lower the shrinkage, the higher the stiffness of the hydrogel, being the resisting force exerted by the matrix against cells stronger.

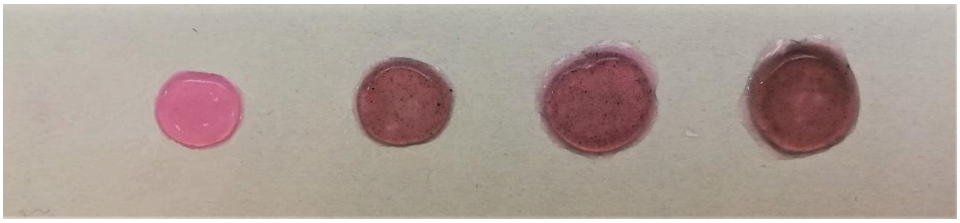


Fig. 5.18 Observation of the shrinkage of collagen (pink) and GO-collagen (at increasing concentrations of GO from the left to the right) hydrogels seeded with $5x10^5$ NIH3T3 cells after 10 days of incubation at 37°C

The effect of the oxide on the stiffness, evaluated through hydrogel's contraction, was in this case more evident with respect to monolayer cell culture. It can be noted that the colour intensity of the 0.5% GO-collagen hydrogel is comparable to that of 2% and 5% GO hydrogels. As a consequence of its much more significant contraction, in fact, the 0.5% GO hydrogel experienced a remarkable compaction, resulting in a hydrogel with a final density much higher than its initial one. Diameters of the hydrogels were measured through the use of a calibre and the shrinkage with respect to the initial size was calculated and expressed as a % shrinkage on a bar chart [fig. 5.19].

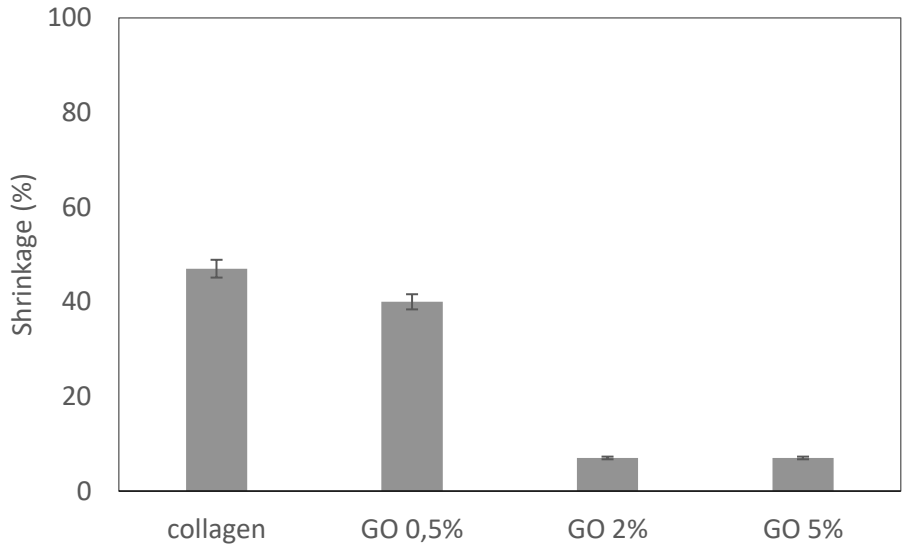


Fig. 5.19 Shrinkage of collagen and GO-collagen hydrogels seeded with $5x10^5$ NIH3T3 cells and evaluated after 10 days of incubation at 37°C

As it can be seen, 0.5% GO-collagen hydrogels got less shrunk than simple collagen hydrogels. Culture dishes were 15 mm in diameter, the collagen hydrogel measured 8 mm while the 0.5% GO-collagen hydrogel measured 9 mm after 10 days of incubation. Both 2% and 5% GO measured 14 mm. According to calculations, hydrogels shrunk down to a 53%, 60% and 93% of their initial diameter respectively. This means that the shrinkage percentages were equal to 47%, 40% and 7% as reported in the graph. This is symptom of a stiffer matrix in case of 0.5% GO-collagen hydrogels. Again, the result is a confirmation of all the outcomes obtained so far: non-exfoliated GO has a reinforcing effect on collagen, even if not particularly significant.

In case of 2% and 5% GO-collagen hydrogels, no conclusions can be drawn on the stiffness of the matrixes. At those concentrations, in fact, cell growth was first inhibited and then totally stopped after a couple of days of incubation [fig. 5.20, 5.21, 5.22, 5.23]. Cell growth was instead clearly visible for collagen and 0.5% GO-collagen hydrogels, especially from day 5 on. As it can be noted, over time fibroblasts took a stress-free stellate morphology due to the possibility for the hydrogels to freely float on the culture medium without any constraint. This shape is different from that observed in case of monolayer cell culture, in which cells adhere to a flat stiff surface. Fibroblasts embedded in 2% and 5% GO kept a round shape instead, which is typical of non-growing cells if observed after days of incubation. In this case, being the concentration of GO quite high, the *in vitro* culture environment was far from mimicking the

physiological environment observed in natural tissues. GO might have caused oxidative stress and acted as physical obstacle to cell growth, leading to cell death.

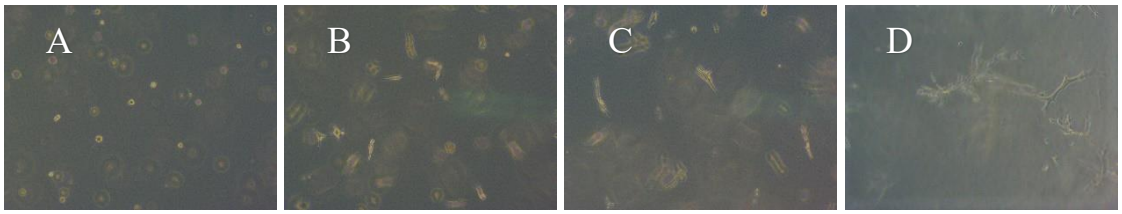


Fig. 5.20 Growth of NIH3T3 cells embedded in collagen hydrogels on day (a) 1, (b) 3, (c) 5 and (d) 10

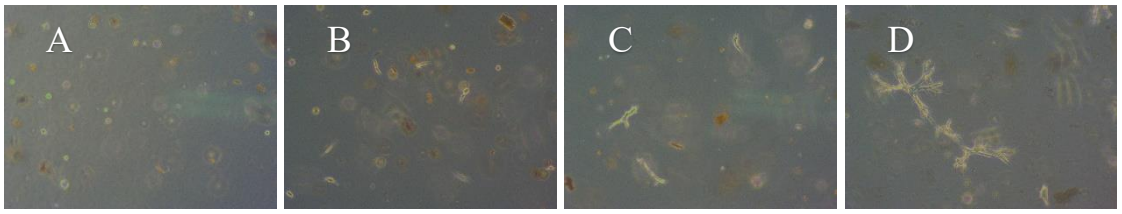


Fig. 5.21 Growth of NIH3T3 cells embedded in 0.5% GO-collagen hydrogels on day (a) 1, (b) 3, (c) 5 and (d) 10

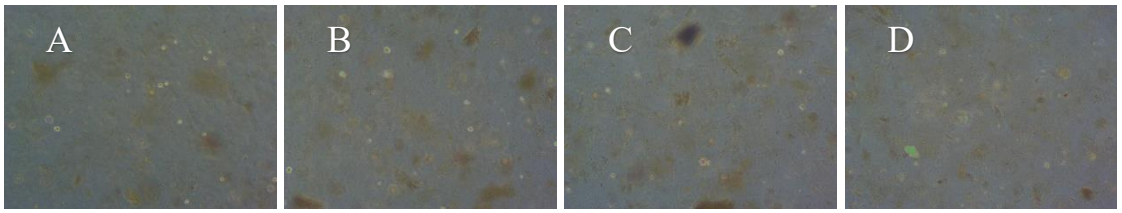


Fig. 5.22 Growth of NIH3T3 cells embedded in 2% GO-collagen hydrogels on day (a) 1, (b) 3, (c) 5 and (d) 10

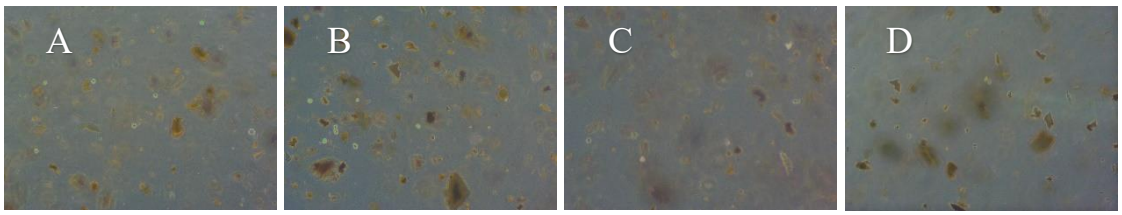


Fig. 5.23 Growth of NIH3T3 cells embedded in 5% GO-collagen hydrogels on day (a) 1, (b) 3, (c) 5 and (d) 10

According to literature [33], in fact, the cytotoxic effect of GO on fibroblasts is dose- and time-dependent. In particular, GO does not exhibit cytotoxicity with doses less than 20 μg/ml, and according to calculations our 2% GO-collagen hydrogels correspond to a concentration of oxide of about 22 μg/ml. This confirms the results reported in literature and explains the reason why cells stopped growing and 2% and 5% GO hydrogels stopped contracting soon. We can

conclude that concentrations equal to or higher than 2% are not suitable for our purposes, which once again makes 0.5% GO-collagen hydrogels the most preferable candidates.

6. Conclusions

The purpose of this study was to use GO as a reinforcer and physical cross-linker for collagen hydrogels and to evaluate the effect of its concentration and size on the secondary structure of collagen and on the stiffness of the resulting nanocomposite hydrogels with respect to simple collagen hydrogels.

GO-collagen nanocomposite hydrogels at increasing concentrations of GO from 0.05% to 5% w/w were successfully fabricated through a modification of the gelation procedure recommended by *Nitta Collagen Inc*®. Both non-exfoliated and exfoliated GO were tested through all the displayed experiments, except for those related to cell culture for which only non-exfoliated GO was used due to lack of time. Results revealed that non-exfoliated GO has a reinforcing effect on collagen hydrogels due to an enhanced interaction among collagen fibrils, while exfoliated GO is responsible for a partial denaturation of the secondary structure of collagen that affects its fibrillogenesis. This is presumably due to the ability of exfoliated GO, which is smaller in size, to intercalate among collagen fibrils affecting the helical structure of collagen and its compaction. A similar result was in fact found by Nishina et al. [37] on the interaction between small-sized GO and siRNA helices. Studies on the secondary structure of collagen were carried out through CD spectroscopy. The different effects of non-exfoliated and exfoliated GO were also confirmed through the evaluation of the water uptake of our hydrogels, which resulted to be respectively lower and higher than that of the reference collagen hydrogels, even if not significantly. Lower amounts of retained water correspond to higher cross-linking densities, being the hydrogels' network more restricted to swell. The higher cross-linking density of non-exfoliated GO-collagen hydrogels was also qualitatively confirmed by SEM imaging. Rheologic measurements revealed that the storage modulus G', indicator for the stiffness of the material, is increased by the presence of non-exfoliated GO and decreased by the addition of exfoliated GO. Overall, non-exfoliated GO has a more effective reinforcing effect on collagen hydrogels than its exfoliated counterpart. For this reason, more detailed conclusions on non-exfoliated GO are illustrated below.

Non-exfoliated GO-collagen hydrogels showed the lowest water uptake for a concentration of GO equal to 0.5%. In particular, the amount of retained water decreased with the increasing concentration of GO up to 0.5% and then started to increase again above this value of concentration. A possible explanation for this behaviour is the presumed co-existence of two opposite effects of GO: an enhancing effect prevailing at low concentrations due to the physical binding of collagen fibrils promoted by the oxide, and a inhibiting effect prevailing at higher concentrations due to the massive presence of oxide flakes that might interfere with the self-assembly of collagen during gelation. This result was confirmed by SEM images, which showed the formation of a denser network in presence of GO at the concentration of 0.5%. Rheologic measurements displayed increments in the values of G' equal to 50% and 12,5% in case of 0.5% and 2% GO respectively, with respect to a simple collagen hydrogel. According to the explanation given for the water uptake results, 5% GO was expected to lead to an even less significant increase of G' than 2% GO. Nevertheless, a 29% increase of G' was in this case found to have occurred. This is probably due to the fact that, despite the inhibition of fibrillogenesis mentioned above, large amounts of GO are also responsible for an overall stiffening which is, though, only given by its intrinsic mechanical strength and not by the cross-linking density of the resulting GO-collagen hydrogels. CD spectra confirmed that the secondary structure of collagen was preserved in presence of non-exfoliated GO. Denaturation of the protein would have led to an undesired loss of its biological activity, which is a

fundamental requirement for cell culture applications. Evaluations of the diameters of cell-seeded hydrogels after 10 days of incubation revealed that hydrogels charged with 0.5% GO were subjected to a 40% contraction, while the reference collagen hydrogels to 47%. This highlights the higher resistance, i.e. the higher mechanical strength, of the formers against the traction forces exerted by cells. For concentrations equal to 2% and 5%, though, nearly no contraction was observed, being equal to barely 7%. Nevertheless, this has not to be intended as a reinforcement of the hydrogels but more likely as a GO-related cytotoxicity of the surrounding environment. By the use of an optical microscope, in fact, no growth of cells was found to have occurred during the 10 days of incubation for these two concentrations of GO. We could then conclude that GO was successfully sterilized and that concentrations above 2% are cytotoxic towards fibroblasts.

Overall, a concentration of non-exfoliated GO equal to 0.5% turned out to be the most effective amount of oxide in terms of cross-linking density, mechanical properties and cell culture suitability of the resulting GO-collagen nanocomposite hydrogels. Our results were not as significant as those observed for other physically cross-linked GO-collagen hydrogels reported in literature in terms of stiffness. It has to be reminded, though, that those hydrogels were differently synthesized and contained much higher amounts of GO, also up 70%. As a consequence, unlike our hydrogels, they exhibited intrinsic-GO-like mechanical properties and a presumably high cytotoxicity risk which would not be suitable for our cell culture purposes and for the fabrication of a tissue equivalent. Moreover, in order to make the concentration of GO the only variable parameter in our experiments and to study its effect on collagen, we focused on hydrogels with a final concentration of collagen fixed to 2.1 mg/ml, which was dictated by the concentration of the purchased solution. Being aware that the mechanical properties of collagen hydrogels have been largely proved to increase with the increasing concentration of collagen itself, better results in terms of stiffness could be already obtained by maintaining the same GO:collagen ratios but using a more concentrated collagen solution.

7. References

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