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Photocrosslinked chitosan hydrogels reinforced with chitosan derived nano-graphene

oxide

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Keywords: chitosan hydrogel, graphene oxide, photopolymerization.

((Abstract: Chitosan and chitosan derived nano-graphene oxide carbon dots are successfully

methacrylated and utilized for fabrication of photocurable hydrogels. The addition of the nano-

graphene oxide (nGO) did not significantly delay the polymer network build-up, but

significantly reduced the storage modulus of the crosslinked network, with important

detrimental effect of the mechanical performance. By replacing nGO with methacrylated M-

nGO, the mechanical performance of the crosslinked polymer network is improved with an

increase of the storage modulus as a function of increasing the M-nGO content in the

photocurable formulation.))

1. Introduction

Polymeric hydrogels can be used in tissue engineering mainly because of their ability to provide

an environment that is similar to hydrated tissues. Furthermore, they can reproduce the

extracellular matrix (ECM) structure and allow cell adhesion and proliferation. Also, hydrogels,

can be designed to gel in-situ, so to behave as scaffold and cell-carrier [1].

1

Chitosan hydrogels have been widely used as a matrix for tissue engineering applications [2-6], showing excellent biocompatibility, enzyme-mediated degradation and non-toxic degradation products.

However, the solubility of chitosan hydrogels at physiological pH and low mechanical properties limit their application. The use of crosslinked chitosan was suggested to overcome these limitations. In light of these considerations, many efforts to prepare functional chitosan by chemical modifications have been reported [7, 8] including synthesis of chitosan-based hydrogels by thermal or photo-initiated copolymerization. The benefits of photo-crosslinking reactions include in-situ formation of the hydrogels using a fast and cheap procedure. Photochemistry is widely known to be a green method and has attracted great attention because it offers many advantages over traditional techniques such as cost efficiency, mild conditions and solvent-free systems [9]. Furthermore, photopolymerization allows the injection of hydrogel precursors followed by rapid curing at the target tissue under mild conditions in a spatiotemporal controlled manner. Valmikinathan et. al reported the use of photo-crosslinkable chitosan-based hydrogels for neural tissue engineering [10]. Specifically, they showed that the crosslinking of chitosan leads to a material that supports cell transplantation. Soraiva et. al. further reported photocurable hydrogels composed of chitosan and methacrylated gelatin [11]. Li et al. obtained photo-crosslinkable chitosan by N-acylation between amino groups and methacrylic anhydride. The methacryloyl groups enhanced chitosan solubility and allowed photocuring reaction [12]. Jun Nie et. al. described the synthesis of promising injectable hydrogels which were prepared from chitosan derivative (EGAMA-CS)/polyethylene glycol dimethacrylate (PEGDA)/N,N-dimethylacrylamide (DMMA) by UV photopolymerization. Such hydrogels show excellent cell adhesion and proliferation properties and could serve as biomaterials for bone tissue engineering [13].

The addition of graphene and graphene oxide to chitosan hydrogels was also reported in the literature. Yang et. al. showed that the tensile strength and Young's modulus of the graphene-

modified chitosan are significantly improved by about 122 and 64%, respectively, with the incorporation of 1 wt % graphene oxide. At the same time, the elongation at the break increased remarkably [14]. Wang et. al. reported the preparation of chitosan/graphene oxide composite films with important improvements on Young's modulus, tensile strength and elongation at break with respect to pristine chitosan [15]. Han et. al. reported that chitosan and graphene oxide could mix with each other homogeneously and the mechanical properties of the asprepared films were improved significantly [16]

Zero dimensional nano-graphene-oxide (nGO) type carbon dots were obtained by microwave assisted carbonization of chitosan [17] or other biopolymers [18]. These carbon dots improve the mechanical performance of genipin crosslinked chitosan hydrogels [19]. The same effect was observed in blends made with bioresorbable scaffolds based on starch [20] and polycaprolactone [21]. These studies show that nGO carbon dots are able to induce mineralization and reinforcing effects in polymer and hydrogel composites. These properties are likewise highly desirable for photocurable hydrogels. Furthermore, functionalization of nGO with photocurable groups would allow them to become part of the photo-cured network. We therefore set out to functionalize chitosan and nGO with methacrylated photocurable groups. Subsequently, their photoreactivity in the presence of a water soluble radical photoinitiator was investigated. The aim of this investigation was the development of photocurable chitosan based hydrogels containing methacrylated-functionalized GO, obtained as well from bio-renewable sources, to improve the mechanical performance of the crosslinked hydrogel.

For photocrosslinking reaction, it was selected an initiator which is active at longer wavelengths, above 350 nm, in order to avoid possible damage of cells in the hydrogel precursor by UV-light which is an application foreseen in the future. The efficiency of the photo-curing process was investigated by means of FT-IR and photorheology and the final hydrogels were fully characterized.

2. Experimental Section

2.1 Materials

Chitosan (CS) and methacrylic anhydride (MA), was purchased from Aldrich. Medium molecular weight Chitosan (CS) had following characteristics as determined previously by us^[19]: number-average molecular weight (Mn) 119 000 g/mol, weight-average molecular weight (Mw) 566 000 g/mol, and molecular weight distribution (MWD) 4.8 as determined by size exclusion chromatography (SEC), viscosity-average molecular weight (Mv) 300 000 g/mol as determined by viscometer and degree of deacetylation ca. 77%, as determined by ¹H NMR. The water soluble radical photoinitiator bismesitoylphosphinic acid (BAPO-OH), was synthesized as previously reported^[22] and used as photoinitiator in the water photocurable formulations. Sulfuric acid (H₂SO₄, 95-98%), nitric acid (HNO₃, 70%), triethylamine (TEA), 99.8%), toluene (anhydrous, sodium biocarbonate (NaHCO3, >99.7%), dimethylformamide (DMF, 299.8%) were purchased from Sigma Aldrich. Acetic acid (CH3CO2H, ≥99.5%) was purchased from Acros Organics. All chemicals were used as received.

2.2 Synthesis of methacrylated chitosan

CS was dissolved overnight in 2 wt% acetic acid solution to prepare a solution with CS concentration of 1.5 wt%. Subsequently, methacrylic anhydride (MA) was added dropwise to achieve MA to amino groups (NH₂) ratios of 0.5, 1, 2, and 4, respectively. The reactions were carried out at 50 °C for 4 h. The resulting solutions were neutralized by adding 20 g of 5 wt% NaHCO₃ solution. The methacrylated chitosan (CS-MA) were dialysized (MWCO=1K) against DI water for 4 days to remove any unreacted reagents. The final products were freeze dried. The products were denoted CS, CS-MA0.5, CS-MA1, CS-MA2 and CS-MA4 based on the MA to amine group ratio during methacrylation. Finally only CS-MA4 were successfully UV-cured.

2.3 Synthesis of graphene oxide

Graphene oxide (nGO) was synthesized based on the previously developed procedure. Briefly, six Teflon fluoro modified vessels with 2 g of CS and 20 ml of H₂SO₄ (0.1 g/ml) were placed into the Milestone flexiWAVE Microwave irradiation chamber with the maximum power of 1200 W. The irradiation temperature was set to reach 220 °C with a RAMP-time of 20 min, and then kept at 220 °C for 2 h. The carbon spheres (C-sphere) presented as the solid black powder were collected through filtration and washing with DI water to reach a neutral pH value. To produced nGO, C-sphere was dispersed in the HNO₃ (10:1 w/v) with sonication at r.t. for 30 min. Then the solution was heated in the oil bath at 90 °C for 1 h with continuous magnetic stirring, after which the cold DI water was added to stop the oxidation reaction, and the brown solid residues, referred as nGO, were obtained after vacuum distillation and freeze-drying process. The detailed characterization of nGO was described in the previous work^[17].

2.4 Synthesis of methacrylated nGO

To synthesize methacrylated nGO (M-nGO), the aforesaid nGO (100 mg) was dispersed in the DMF (1 mg/ml) with addition of 10 ml of TEA as the acid binding agent. 30 min of sonication was applied on the mixed solution to obtain better nGO dispersity. After that, 50 ml of mixed solution containing 5 ml of MA and 45 ml of DMF was added dropwise into the nGO solution, with the constant temperature of 70 °C in an oil bath for 24 h. The M-nGO was obtained via rotary evaporation and further precipitation in the toluene. Finally, M-nGO was purified by drying in the vacuum oven at room temperature for 3 days before use.

2.4 Preparation of photocrosslinkable chitosan-based formulations

The methacrylated CS (grafting ratio 4) was dissolved in water at a concentration of 10 mg/ml. The BAPO-OH photoinitiator was added at a content of 2 wt% with respect to the CS-MA content. The formulation was irradiated with a visible lamp (Hamamatsu LC8 with visible bulb

and a cut-off filter below 400 nm equipped with 8 mm light guide) for 3 minutes. The CS-nGO hydrogels were prepared by adding to the above-mentioned formulations 1, 2 or 5 wt% nGO filler with respect to the CS-MA content. The samples were denoted CS-MAX/nGOY, where X gives the MA amine group ration during the methacrylation and Y gives the wt% of nGO with respect to CS-MA content. For comparison composite hydrogels with methacrylated nGO were also prepared and denoted CS-MAX/M-nGOY according to same principal.

2.5 Characterization

The methacrylation of chitosan and nGO was confirmed by FTIR and NMR. The FTIR spectra were recorded by a PerkinElmer Spectrum 2000 FTIR spectrometer (Norwalk, CT) equipped with an attenuated total reflectance (ATR) crystal accessory (Golden Gate). The resolution was set to be 4 cm⁻¹ and scan range was in between 600 and 4000 cm⁻¹. For the ¹H NMR measurement, 15 mg/ml methacrylated CS solution (dissolved in D₂O) was prepared. The NMR spectrum was acquired by using a Bruker Avance DPX-400 NMR instrument operating at 400.13 MHz. The photopolymerization process was followed by measuring the methacrylated double bond conversion by FTIR spectroscopy during irradiation, employing a Thermo-Nicolet 5700 instrument. The double bond conversion was calculated by monitoring the decrease in the absorbance of the peak centered at around 840 cm⁻¹. The formulations were cured with a visible lamp (Hamamatsu LC8 with visible bulb and a cut-off filter below 400 nm equipped with 8 mm light guide).

Photo rheological tests were performed by using an Anton Paar rheometer (Physica MCR 302), equipped with a portable visible lamp (Hamamatsu LC8 with visible bulb and a cut-off filter below 400 nm equipped with 8 mm light guide). The strain (%) and angular frequency (rad/s) were set constantly as 0.1% and 60 rad/s, respectively. For each sample, the storage modulus as a function of time (s) was recorded, with the light source turned on after 2 min in order to stabilize the system. All the measurements were carried out at r.t.

Swelling tests were performed on dried cylinder-shaped hydrogel samples with 4 mm in diameter that were gently incised and immersed into 10 ml of DI water for 48 h to reach the swelling equilibrium. The tests were performed at r.t., and for each type of hydrogel, three-parallel experiments were carried out. The swelling ratio at equilibrium (Qeq) was determined according to Eq. (1)

$$Qeq = (meq-m0)/m0$$
 (1)

Where m0, and meq represent the weight of the hydrogel at initial state and at equilibrium, respectively.

The storage modulus the crosslinked hydrogels were assessed by TA Discovery Hybrid 2 (DHR-2) rheometer equipped with an 8 mm stainless steel Peltier plate at room temperature. The applied strain rate was set to 1% from the linear viscoelastic region, and the oscillation frequency rate range was from 0.01 to 10 Hz.

3. Results and Discussion

3.1 Functionalization of chitosan and nano-graphene oxide by photocurable groups

To fabricate photocurable composite hydrogels, chitosan and chitosan derived nano-graphene oxide (nGO) was functionalized by methacrylation (Figure 1). The strategy was to use this methacrylated nGO, namely M-nGO, to enhance the mechanical properties of photo-curable chitosan hydrogels. By methacrylation of both components to give CS-MA and M-nGO we hoped to enable a covalent linkage between the chitosan matrix and the carbon filler.

NMR (Figure 2) and FTIR (Figure 3) confirmed the successful methacrylation of chitosan. The degree of methacrylation was determined by ¹H-NMR spectroscopy. The ¹H NMR spectra show the characteristic peaks of CS glucosamine rings as shown in the pink frame (around 3 - 4 ppm, 6H). The grafting of MA groups to CS was confirmed by the appearance of methylene protons

(around 5.5 -6.1 ppm, 2H, grey frame) and methyl protons (1.8 - 1.9 ppm, 3H). The degree of methacrylation was determined by integration of the intensity of the signals and calculated to be approximately 9 %, 10 %, 11 %, or 20 % for the synthesis with MA to amino group ratios of 0.5, 1, 2, and 4, respectively. The FTIR spectra further prove the successful grafting of MA to CS as evidenced by shifting of the N-H stretching vibration to a lower wavenumber indicating N-acylation. The FTIR spectra show no absorptions of ester bonds proving that the primary amine groups of chitosan are the main reaction sites.

FTIR was also utilized to confirm the methacrylation of nGO. Figure 4 displays the comparison of the FTIR spectra of MA, nGO and M-nGO showing the presence of methacrylic double bond in the functionalized M-nGO.

3.2 The fabrication of photocurable hydrogels and composite hydrogels

The photo-crosslinking reaction of modified chitosan with the highest degree of methacrylation CS-MA4 was investigated by means of FT-IR and photorheology analysis. Water-soluble photoinitiator was added to promote radical-induced photopolymerization.

In Figure 5 the FT-IR spectra before and after curing is reported for chitosan. The data show a complete acrylic double bond conversion, with the disappearance of the C=C double bond peak centred at 845 cm-1, after 3 minutes of irradiation with visible light. This is a clear indication of the high reactivity of the methacrylated chitosan, as was also suggested by photorheological studies (see curves reported in Figure 6).

Photorheological studies were likewise performed on methacrylated chitosan, CS-MA4, see Figure 6. To evaluate the possible inhibition of photocuring by nGO, the photorheology after addition of 2 and 5 wt% of unmodified nGO was studied as well and compared to the photorheology of plain CS-MA4. The storage modulus (G') as a function of irradiation time is reported in Figure 6 for the investigated chitosan-based formulations.

The data collected from photorheological investigations agree with the FT-IR data, showing the starting of network formation within 3 minutes of irradiation. When GO was dispersed in the photocurable formulation no significant delay of the formation of a crosslinked network was observed. But a detrimental effect was measured on the build-up of storage modulus: the G'-storage modulus decreased with increasing content of nGO in the formulations.

This is an indication of a lower crosslinking density, suggesting a decrease of the acrylic double bond conversion upon irradiation by increasing the nGO content in the formulation. This can be attributed to the strong screening effect of nGO towards visible light, which leads to a significant reduction of photogenerated reactive radicals which affects the final methacrylic double bond conversion.

The photorheological studies are in good agreement with the swelling degree of the crosslinked methacrylated chitosan compared to the swelling degree of the crosslinked chitosan containing nGO. The swelling data are collected in Figure 7. It is evident that there is an increase in the swelling ratio as the nGO content is increased in the photocurable formulations. This can be ascribed to the decreased crosslinking density as a function of increasing the filler content, attributed to the screening effect during photopolymerization, which reduced the acrylic double bond conversion. A lower crosslinking density will allow a higher swelling ratio, but consequently also a lower modulus and mechanical performance of the crosslinked hydrogel, as shown by photorheological test.

To improve the mechanical performance of the crosslinked chitosan-based hydrogels, without compromising the achieved increased degree of swelling, the methacrylated M-nGO was dispersed in the photocurable chitosan formulation, to enable covalent attachment of M-nGO to the polymeric network.

In Figure 8 the rheological properties are reported for crosslinked chitosan hydrogels (CS-MA4) and compared with the rheological properties recorded for the hydrogels containing either non-methacrylated nGO at 2 wt% and 5 wt% (CS-MA4/nGO2 and CS-MA4/nGO5 respectively) or

methacrylated M-nGO at 1 wt%, 2 wt% and 5 wt% (CS-MA4/M-nGO1, CS-MA4/M-nGO2 and CS-MA4/M-nGO5, respectively).

As stated above, it is evident that the presence of non-methacrylated nGO in the photocurable formulations induces a drop of the storage modulus with increasing nGO content in comparison with hydrogels prepared from plain methacrylated CS. On contrary, when methacrylated M-nGO was added instead, an important enhancement of the storage modulus was achieved. The increase of the storage modulus becomes more significant with increasing M-nGO content. This can be explained by the reinforcing effect obtained by the additional covalent cross-linkages between the methacrylic groups on the M-nGO surface and CS-MA. The data give clear evidence that methacrylation of nGO can more than counterbalance the screening effect of nGO towards visible light leading to an overall reinforcement and improvement of the mechanical performance of photo-crosslinked chitosan hydrogels.

4. Conclusions

Chitosan and chitosan derived nano-graphene oxide carbon dots were successfully methacrylated and utilized for fabrication of photocurable hydrogels. The visible light irradiation of methacrylated chitosan was investigated showing the high reactivity with the formation of the polymeric network within 3 minutes of irradiation. The addition of the non-methacrylated nGO did not significantly delayed the polymer network build-up, but significantly reduced the storage modulus of the crosslinked network, with important detrimental effect of the mechanical performance. This was attributed to the screening effect of nGO against visible light. By replacing nGO with methacrylated M-nGO, it was, however, possible to enhance the mechanical performance of the crosslinked polymer network with an increase of the storage modulus as a function of increasing the M-nGO content in the photocurable formulation. This was attributed to the formation of chemical bonds among the filler and the polymeric network. In conclusion, this paper shows the important feasibility of

improving the mechanical performance of chitosan-based photocured hydrogels by dispersing methacrylated functionalized nGO filler from bio-renewable sources.

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Figure 1. Reaction scheme of chitosan methacrylation

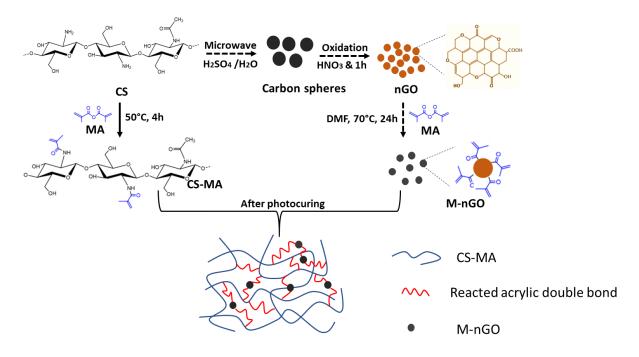


Figure 2. NMR spectra of methacrylated CS with different MA to amino group feed ratios

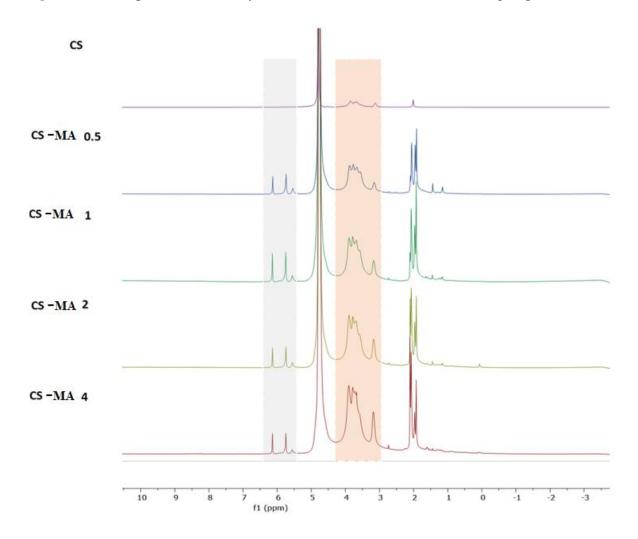


Figure 3. FT-IR spectra of methacrylated CS with different MA to amino group feed ratios

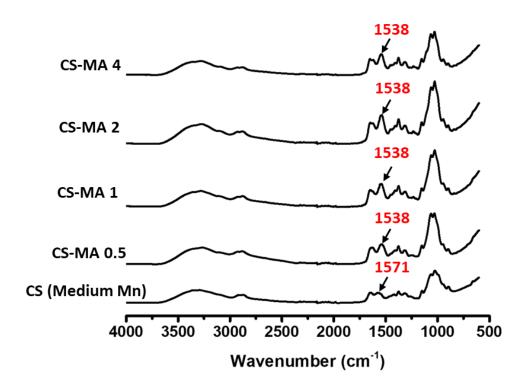


Figure 4. FT-IR spectra of MA, nGO and M-nGO.

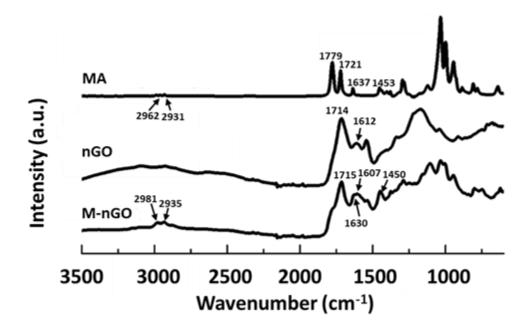


Figure 5. FT-IR before and after irradiation of the pristine chitosan-based formulations

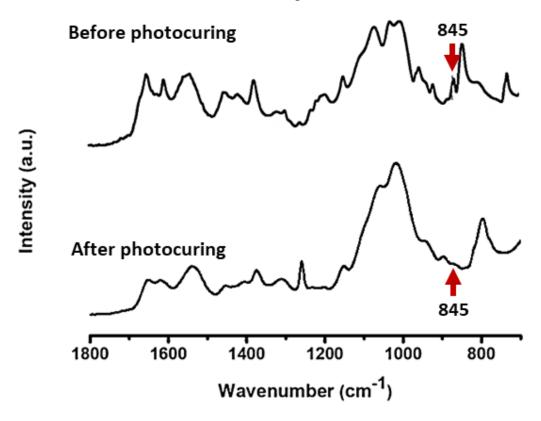


Figure 6. Photorheological storage curves (G') as a function of irradiation time for the methacrylated chitosan (CS-MA4), and for the formulations containing 2 wt% (CS-MA4/nGO2) and 5 wt% (CS-MA4/nGO5) of nGO, respectively.

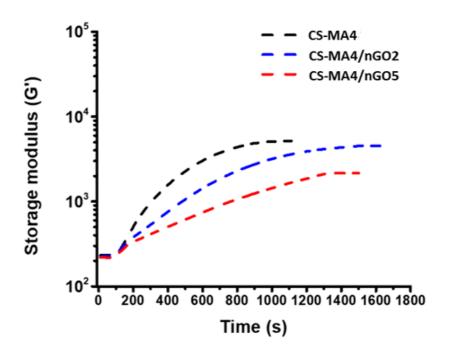


Figure 7. swelling ratio of the pristine chitosan and chitosan-graphene oxide crosslinked hydrogel.

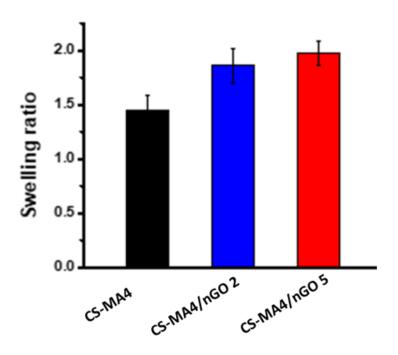
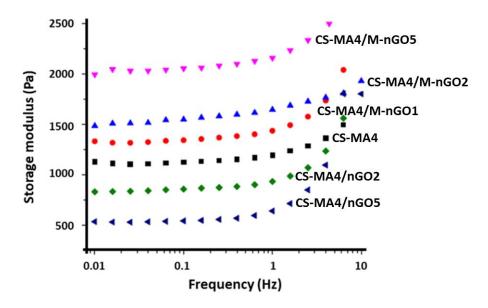


Figure 8. Rheological curves of the photocured chitosan-based hydrogels.



Photoinduced chitosan hydrogels reinforced with chitosan derived nano-graphene oxide

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Keyword chitosan hydrogel, graphene oxide, photopolymerization.

Chitosan and chitosan derived nano-graphene oxide carbon dots (nGO) are successfully methacrylated and utilized for fabrication of photocurable hydrogels. Mechanical performance of chitosan-based photocured hydrogels are by dispersing methacrylated functionalized nGO.

ToC figure

