Low-cost alginate-based nanocomposite films showed high antiviral activity

Isaías Sanmartín-Santos[1,¶], Sofía Gandía-Llop[1,2,¶], and Ángel Serrano-Aroca[2,*]

[1]Structural and Computational Biochemistry Lab, Centro de Investigación Traslacional San Alberto Magno, Universidad Católica de Valencia San Vicente Mártir, c/Quevedo 2, 46001 Valencia, Spain.

[2]Biomaterials and Bioengineering Lab, Centro de Investigación Traslacional San Alberto Magno, Universidad Católica de Valencia San Vicente Mártir, c/Guillem de Castro 94, 46001 Valencia, Spain.

[¶] These authors contributed equally.

*Corresponding author: angel.serrano@ucv.es; Tel.: +34-963637412 (ext. 5256)

Abstract

Due to the current COVID-19 situation caused by the spread of the new SARS-Cov-2 coronavirus, new alternative materials with antiviral activity are encouraged to be fabricated by the World Health Organization for present and future pandemics. Previous studies of low-cost alginate-based nanocomposite films produced with very low amounts (0.1% *w/w*) of carbon nanomaterials such as graphene oxide (GO) or carbon nanofibers (CNFs) have shown very low transparency reduction, enhanced mechanical performance, improved water diffusion and wettability, and similar biological properties than neat alginate in terms of cell adhesion and non-cytotoxicity. However, only the nanocomposite containing CNFs have shown antibacterial activity. Herein, the antiviral properties of these nanocomposite biomaterials are explored for the first time in literature using a double-stranded DNA viral model. The results of this study showed that neat calcium alginate films possess antiviral activity and the incorporation of that low percentage of CNFs significantly enhanced its antiviral action from ~55.6% to 96.33% inhibition after 48 hours. Nevertheless, the addition of this minuscule amount of GO did not improve the antiviral activity of calcium alginate. Nonetheless, both antiviral composite biomaterials possess excellent physical and biological properties with great potential in biomedical applications.

Keywords: graphene oxide, carbon nanofibers, calcium alginate, nanocomposite films, antiviral activity

1. Introduction

Sodium alginate (SA) has been authorized by the US Food and Drug Administration for human biomedical applications due to its excellent properties such as biodegradability, renewability, cost-effectiveness, non-toxicity and biocompatibility[1]. This biopolymer can be cross-linked with Ca^{2+} cations to form hydrogels [2] with physical properties such as mechanical performance, water diffusion and wettability that can be enhanced by the incorporation of low amounts of carbon nanomaterials (CNMs) such as graphene oxide (GO) [3–5] or carbon nanofibers (CNFs) [6,7]. These nanocomposites possess similar biological properties than neat calcium alginate in terms of cell adhesion[8] and cytotoxicity[9,10]. CNFs are hollow carbon filaments of one dimension with antibacterial activity in pure form and when incorporated into calcium alginate[9] as well as GO[10]. However, in these previous studies, the calcium alginate/GO nanocomposite films required a higher percentage of CNMs (from 0.5% *w/w*) in contrast to the calcium alginate/CNFs nanocomposite films (from 0.1% *w/w*) to acquire antibacterial activity. The antiviral properties of calcium alginate has been hardly explored [11,12]. However, as far as we know, the antiviral activity of calcium alginate with carbon nanofibers and graphene oxide has never been studied before. Thus, herein, we explore the antiviral activity of these nanocomposites using a tailed double-stranded DNA viral model [13].

2. Materials and Methods

2.1. Materials

Sodium alginate (Panreac AppliChem, Darmstadt, Germany), calcium chloride (anhydrous, granular, ≤7.0 mm, ≥93.0%, Sigma-Aldrich, Saint Louis, Missouri, USA),

graphene oxide (Ref: 796034, powder, 15-20 sheets, 4-10% edge-oxidized, Sigma-Aldrich, Saint Louis, Missouri, USA) and carbon nanofibers (Ref: 13/0248, Graphenano, Yecla, Spain) were used as received.

2.2. Synthesis

Alginate nanocomposite films of approximately 0.25 g were prepared with a composition of 99.9% *w/w* of SA and 0.1% *w/w* of GO or CNFs following a recently reported new engineering route to produce more homogenous alginate-based composites with enhanced physical properties [4]. Thus, mixing of GO/SA or CNFs/SA in 22 ml of distilled water was performed by magnetically stirring for 1 hour at room temperature (26±0.5ºC). After that, another aqueous solution containing 6% (with respect to the SA mass) of $CaCl_2$ in 10 ml of distilled water was mixed with the GO/SA or CNFs/SA aqueous solution for 10 additional minutes. Thus, thin films were produced in Petri dishes after 24 h of drying at 37±0.5ºC in an oven by solvent evaporation. Finally, the films were cross-linked by immersion in an aqueous calcium chloride solution (2% *w/v*) for 2 hours. After rinsing with distilled water, the films were vacuum dried at 60°C±0.5ºC. The calcium alginate films without CNMs were produced following the same chemical procedure but without adding any CNMs. These films will be referred to as Alginate, GO0.1% and CNFs0.1% films.

2.3. Characterization

2.3.1. Biopolymer characterization

The sodium alginate used in this study was characterized by nuclear magnetic resonance (NMR), high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) and size exclusion chromatography with multi angle light scattering detection (SEC-MALS) by the NOBIPOL research group at the Norwegian University of Science and Technology (NTNU).

2.3.2. Electron microscopy

The GO nanosheets and CNFs were observed by high-resolution transmission electron microscopy (HR-TEM) in a JEM 2100F (JEOL, Japan) 200 kV electron microscope. The sample preparation consisted of dispersing a very low amount of CNMs in dichloromethane placed inside an ultrasound bath for ten minutes and subsequent drying at ambient temperature before HR-TEM observation. A JEM-1010 (JEOL, Japan) 100 kV transmission electron microscope (TEM) was utilized to observe the CNFs and GO nanomaterials incorporated into the calcium alginate films. Ultrathin sample preparation with sections of 60 nm was performed with a Leica Ultracut UC6 ultramicrotome (Leica Mikrosysteme GmbH, Austria) and a Diatome diamond knife (Diatome Ltd., Switzerland). The specimens were placed on TEM grids (300 mesh) coated in carbon film.

2.3.3. Antiviral activity tests

The *Escherichia coli* B, living, bacteriophage host (Reference: 12-4300) and the coliphage T4r (Reference: 12-4335) from Carolina (Burlington, North Carolina, USA)

was used for the antiviral tests. The sample films of alginate, GO0.1% and CNFs0.1% were cut into 1 mm diameter discs and sterilized by washing the films during 30 minutes in a beaker with 50 µl of 70% ethanol under magnetic stirring at 450 r.p.m. with fresh ethanol every 10 minutes. After that, the discs were dried at room temperature and exposed to ultraviolet light 1 hour per each side of the disc. Finally, the antiviral activity of the films was analyzed by a "contact test" of the bacteriophages with the discs in 96-well plates for 0, 18 and 48 hours. Several 1/10 serial dilutions from the original concentrate phage stock of about $1x10^9$ plaque forming units (PFU) per ml were tested and the 10^{-5} dilution was chosen from this stock as optimal for our experiments. Roughly, 50 µl of this dilution contains about 500 PFU. The dry film disc was placed in a precision balance and drops of phosphate buffer saline (PBS) solution were added with a volumetric micropipette until the film was saturated without liquid excess. The adequate volume for the saturation point was determined gravimetrically by the increase in weight and corresponds to 44 µl of liquid by disc. For the "contact test", 44 µl of the 10^{-5} phage dilution was added to each disc and subsequently sealed the 96-well plate with plastic film to prevent evaporation during the assay time. After each time, the wells were filled with 500 µl of PBS for washing. The washes were made by adding the buffer and performing 20 resuspensions throughout the surface of the disc with the help of the volumetric micropipette. Once the resuspensions were finished and the discs were extracted using a sterile forceps, and stored with their corresponding liquid in tubes of 15

µl and sonicated for 5 minutes at an amplitude of 10. Then, the tubes were vortexed for 1 minute. Once this process was carried out, we proceeded to the titrations of all the discs of the samples of contact at the different times. Six discs of each material were used to provide reproducible results at the three different contact times.

The determination of the infectious phage particles was performed by the “double layer” assay method. Briefly, the phage aliquots extracted from the disc were serial diluted (1/10 dilution factor) in PBS. An aliquot of 100 µl of each of the dilution of the phage were mixed with 150 µl of bacterial culture and 3 ml of the "top agar" (0.75% w/w agar in LB medium) preheated in a bath incubator at 45ºC. The *E. coli* B culture used was grown overnight until reaching an optical density of 1.0 at 600nm. The samples are softly shaken and subsequently they are poured over Petri dishes with the “bottom agar” (1.5% *w/w* agar in LB medium). The viable viral particles PFU were calculated from the plaques appearing on the soft agar surface stained with crystal violet.

2.3.4. Statistical analysis

The statistical analyses were performed by ANOVA followed by the Tukey's posthoc test ($^{***}p > 0.001$) with the GraphPad Prism 6 software.

3. Results and Discussion

3.1. Characterization of the sodium alginate

The results of the sodium alginate characterization by NMR, SEC-MALS and HPAEC-PAD showed: a guluronic acid content of 43% and a proportion of alginate consisting of guluronic acid in blocks of dimers and trimmers of 27 and 23% respectively, a weight-

average (M_w) and a number-average (M_n) molecular weight of 379.5±9.5 and 170.7±3.1KDa respectively.

3.2. Disc morphologies by electron microscopy

The GO and CNFs in the form of powder were observed by high-resolution transmission electron microscopy (HR-TEM) and showed a morphology of irregular nanometric sheets and micrometer length hollow fibers with a wide range of nanometric diameters respectively (see Figure 1 (a&b)). The EDS results of the CNFs and GO showed C/O ratios of 31.3 and 15.4, respectively in a previous study performed with the same CNMs[8]. The Raman spectra of these CNMs were also analyzed in that study (see whole spectra in the supplementary material of reference [8]) and exhibited an I_D/I_G ratio of 0.92 for the hydrophilic 2D material (GO nanosheets) and 1.51 for the hydrophobic 1D filamentous CNFs due to their higher degree of molecular disorder[14]. The TEM micrographs (Figure 1(c&b)) shows that the GO nanosheets and CNFs (dark phase) are embedded and randomly distributed in the alginate polymer matrix (clear phase).

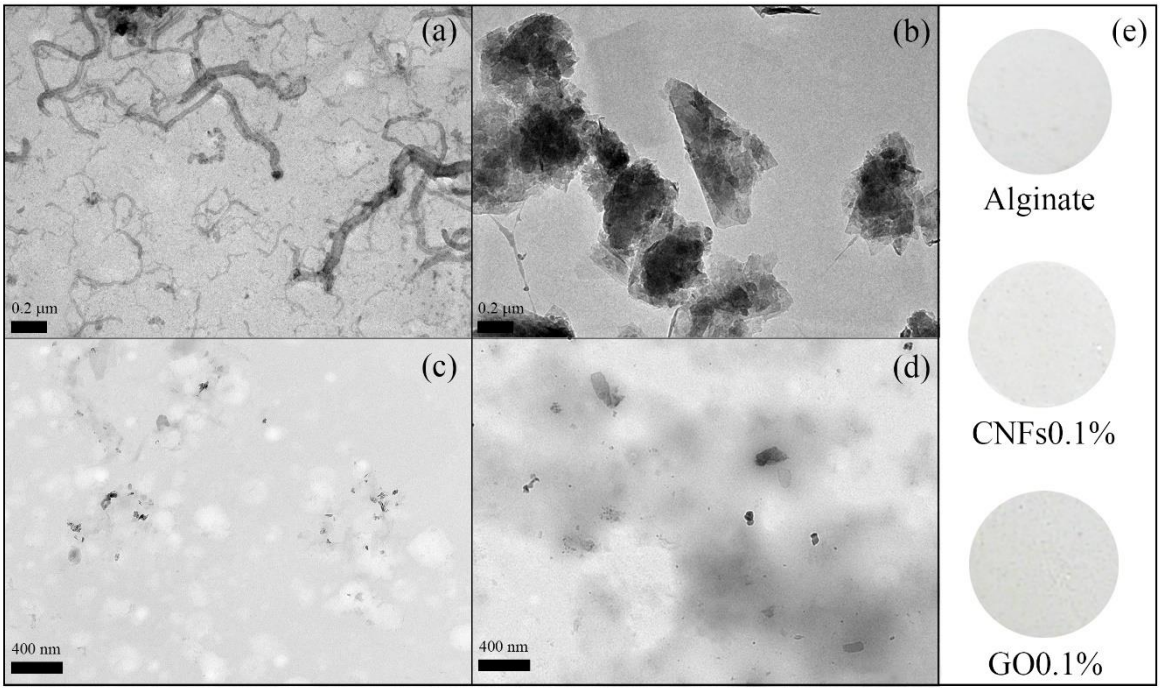

Figure 1. HR-TEM of carbon nanofibers (a), graphene oxide nanosheets (b), calcium alginate films with 0.1% *w/w* of carbon nanofibers (c), calcium alginate films with 0.1% *w/w* of graphene oxide (d) and digital photograph of the films (e).

Figure 1(e) shows that these antiviral nanocomposite materials produced a negligible reduction of transparency in good agreement with previous studies performed by our research group neither with the incorporation of carbon nanofibers[7] nor with that of graphene oxide [10].

3.3. Antiviral properties

The antiviral properties of calcium alginate with a low percentage of carbon nanofibers and graphene oxide were studied here for the first time in literature. Thus, Figure 2 shows

the plaque forming units measured in the antiviral tests performed after 0, 18 and 48 hours of contact with the coliphage T4r.

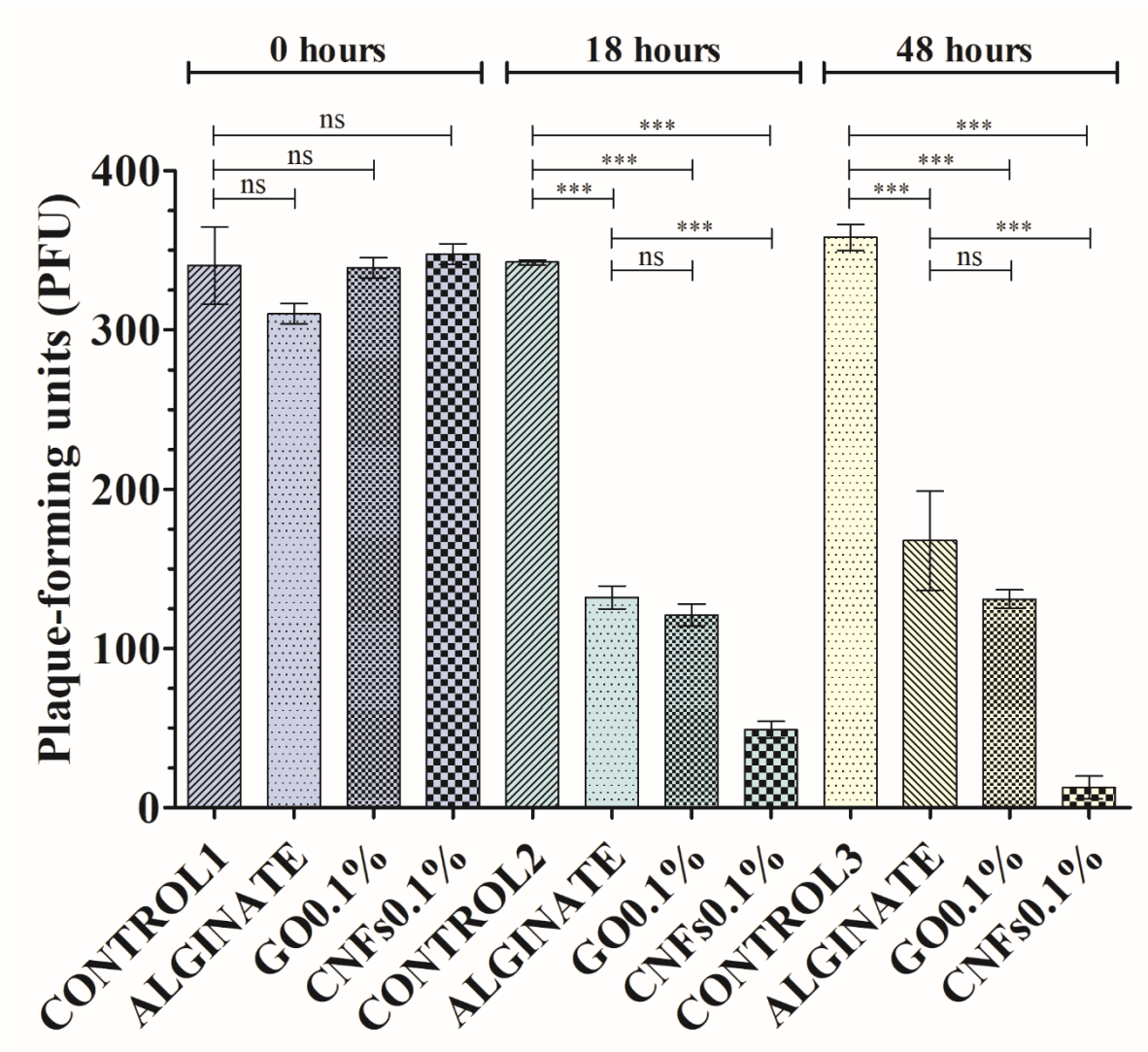

Figure 2. Antiviral activity results of control, and films of alginate, alginate/graphene oxide (GO0.1%) and alginate/carbon nanofibers (CNFs0.1%) at 0, 18 and 48 hours of contact with the coliphage T4r viral model. ***$p > 0.001$; ns: not significant

The antiviral activity of these samples at 0 hours is not statistically significant as expected. These results at time zero indicate that the virus is able to go out of the sample films after the sonication and vortex treatment, and some of contact time is required for them in order to be deactivated. However, when the coliphages T4r are in contact with the alginate film for 18 hours, a decrease of ~56-55.6% in the viable phage counts after 18 hours occurs (see Figure 2 and 3).

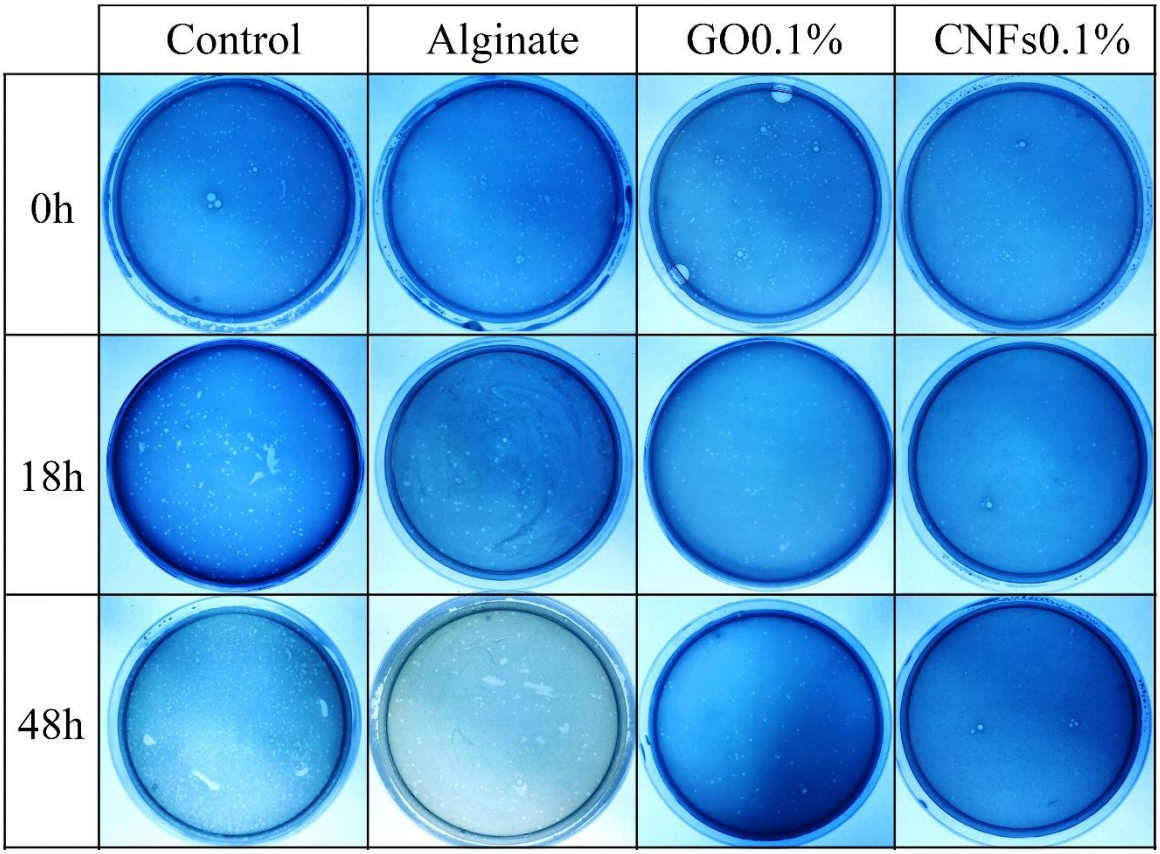

Figure 3. Representative titration plaques of coliphages T4r : antiviral tests of the films of alginate, alginate/graphene oxide (GO0.1%) and alginate/carbon nanofibers (CNFs0.1%) at 0, 18 and 48 hours of contact with the coliphages T4r.

This antiviral action of calcium alginate did not improve either after more time (48 hours), or with the incorporation of graphene oxide (see Figure 2 and 3). However, the calcium alginate films with 0.1% of carbon nanofibers achieved a viral inactivation of ~85.51 after 18 hours, which was enhanced up to 96.33% after 48 hours. These antiviral results are in good agreement with the antibacterial tests performed with the same calcium alginate films produced with 0.1% *w/w* of GO[10] or CNFs[9], which also showed only enhancement of antimicrobial activity with the incorporation of CNFs. Furthermore, these antiviral composite materials have a very low reduction of transparency [7,10] and thus, they can be very promising for biomedical applications with transparency requirements such as ophthalmology and odontology.

4. Conclusions

We have demonstrated that a minuscule amount of carbon nanofibers (0.1% *w/w*) can significantly enhance the antiviral activity of calcium alginate contrary to graphene oxide. Nonetheless, both alginate-based nanocomposites possess antiviral activity, and enhanced physical and biological properties which render them great potential in biomedical applications with antimicrobial requirements.

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Conflicts of Interest

The authors declare no conflict of interest.

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