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Injectable supramolecular hydrogels as delivery system of siRNA loaded PLGA nanoparticles

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***Abstract*— The potential of gene technology to revolutionize disease management by targeting their underlying causes is undeniable. Nevertheless, the efficient administration of these treatments through physiological barriers is challenging. The aim of this research was to evaluate the potential of a supramolecular (SM), bioerodible and injectable hydrogel, composed of an *ad-hoc* custom synthesized poly(ether urethane) (PEU) and commercial *α*-cyclodextrins (CDs), as a conduit for localized gene therapy delivery. To this aim, poly(lactic-*co*-glycolic acid) (PLGA) nanoparticles (NPs) encapsulating small interfering RNA (siRNA) were designed and loaded within the SM hydrogels. PLGA NPs were prepared following a well-defined protocol, resuspended in a 14% w/v CD aqueous solution, and characterized through Dynamic light scattering (DLS) and Phase Analysis Light Scattering (PALS) techniques. The particle size was measured to be around 200 nm (PDI < 0.05), with a negative zeta potential (-20 mV). The encapsulation efficiency (*ca.* 55%) was quantified through RiboGreen assay. The NPs were incorporated into the SM hydrogels and a comparative analysis of unloaded and NPs loaded hydrogels showed no significant changes in the gelation kinetics, while rheological properties were slightly affected by the presence of NPs. Release kinetics found comprehensive elucidation through a fluorescence-based assay, revealing a uniform release curve, with complete release achieved after 48 hours of incubation. Both the hydrodynamic diameter and PDI of the released particles were significantly higher than the freshly prepared NPs, probably because the released NPs were entangled or mixed with gel erosion products. Cell culture experiments using the H1299 cell line evidenced NP uptake without translating the expected gene silencing outcomes. Collectively, this study underscored the promising utility of SM hydrogels as vehicles for gene delivery applications, premised upon their injectability, biodegradability, and favorable release profiles.**

***Keywords*— supramolecular hydrogels, polyurethane, nanoparticles, gene therapy**

I. Introduction

Gene therapies have demonstrated superior clinical outcomes compared to traditional treatments. By delivering a functional version of the defective gene or directly modifying the defective gene, gene therapy offers treatments for monogenic diseases that would otherwise be untreatable through conventional methods. However, an essential obstacle concerning the application of DNA and RNA as therapeutics lies in effectively delivering them into the cell nuclei of specific tissues [1]. For this reason, nanocarriers are widely employed to encapsulate and transport genetic material into targeted cells. Among non-viral nanocarriers, PLGA plays a crucial role due to its low toxicity and favorable safety profile, being FDA approved [2]. Since PLGA is neutral, it does not form polyplexes with nucleic acids like RNA; instead, RNA can be loaded into PLGA nanoparticles or microparticles. To enhance encapsulation and transfection efficiency, a small amount of cationic polymer such as polyethylenimine (PEI) can be incorporated into PLGA NPs, while minimizing toxicity compared to the use of PEI alone.

Although nanocarriers have improved delivery, many challenges, such as targeting specific tissues, efficient encapsulation, overcoming barriers to cell uptake, and minimizing toxicity, still arise. To address these challenges, injectable hydrogels have emerged as promising platforms for the efficient delivery of nanocarriers. This class of hydrogels demonstrates shear-thinning and self-healing properties. The payloads can be homogeneously loaded into the hydrogel formulation while it is still in the liquid form, before gelation. Moreover, shear-thinning and self-healing properties of these hydrogels enable smooth injection through needles without clogging, and allow for recovery to their initial state upon injection. These characteristics make them highly suitable for controlled and prolonged release of various RNA nanocarriers, while being delivered to challenging or inaccessible sites without the need for surgery.

In this work, we assessed the potential of a newly developed supramolecular hydrogel based on a custom-synthesized PEU and CDs [3] as carrier for the localized delivery of siRNA-loaded PLGA nanoparticles.

II. Materials and Methods

A. PEU synthesis and characterization

The PEU was synthesized through a two-step polymerization process, according to a previous protocol [3]. During the first step, Poloxamer® 407 (P407) and 1,6-hexamethylene diisocyanate (added in a molar ratio of 2:1) reacted at 80 °C for 2.5 hours under stirring in presence of dibutyltin dilaurate as catalyst. During the second step (60 °C, 1.5 hours) the chain extender 1,4-cyclohexanedimethanol was added at a 1:1 molar ratio with respect to P407. The resulting polymer was characterized through infrared (IR) spectroscopy to assess the formation of urethane bonds. Meanwhile, the molecular weight distribution profile of the polymer was obtained through size exclusion chromatography (SEC).

B. PLGA NPs preparation and characterization

PLGA NPs were prepared following a protocol known as "emulsion/solvent diffusion technique" [4], where the organic phase is represented by a PLGA solution (1% w/v in methylene chloride), while the internal water phase is represented by an aqueous solution of siRNA (2.5 nmol/mL) and PEI (branched, 25 kDa), with an N/P ratio (Nitrogen from

PEI to Phosphate groups from siRNA) of 10. Instead, the external water phase is represented by a poly(vinyl alcohol) aqueous solution and ethanol (1:1 volume ratio). The NPs were collected through centrifugation and resuspended either in highly purified water (HPW) or in CD solution at 14 % w/v concentration in HPW.
The hydrodynamic diameter and PDI were determined by Dynamic Light Scattering (DLS). The zeta potential was measured by means of Phase Analysis Light Scattering (PALS).
The amount of siRNA encapsulated within the PLGA NPs was quantified through the fluorescence-based Quant-it RiboGreen RNA Assay (Thermo Fisher Scientific), after NPs dissolution with dimethyl sulfoxide.

C. Hydrogel preparation

In order to prepare the supramolecular hydrogel, the PEU was solubilized in HPW at 4 °C. In parallel, a CD solution (14 % w/v) in HPW was prepared and divided into two equal parts: the former was exploited to resuspend the PLGA NPs, while the remaining part was left as such. The two CD solutions were gently pipetted together and subsequently mixed with the PEU solution by vortexing. The resulting mixture was stored overnight at room temperature (RT) until complete gelation. PEU and CD aqueous solutions were mixed to achieve final concentrations of 0.9% and 10% w/v, respectively. PLGA NPs were loaded at *ca.* 15 mg/mL. Control samples were also prepared using the CD solution without NPs.

D. Hydrogel characterization

Hydrogel gelation potential and kinetics were qualitatively evaluated by the tube inverting test. Then, rheological tests were conducted to assess NPs contribution to hydrogel properties. Three tests were performed: (i) strain sweep test within the strain range from 0.01% to 500%, at 37 °C and 1 rad/s; (ii) frequency sweep test with angular frequency varying between 100 and 0.1 rad/s, at 0.1% strain (within the linear viscoelastic (LVE) established by strain sweep test) and 37 °C; (iii) self-healing strain tests through application of three cycles of low (0.1% for 120 s) and high (100% for 60 s) strain, at 37 °C and 1 rad/s. The last applied deformation was the low strain in order to register the residual mechanical properties.
The potential changes in the swelling and dissolution kinetics of PLGA NPs-loaded hydrogels were evaluated through stability tests at 37 °C within a physiological aqueous milieu. The hydrogel loaded with PLGA NPs was compared to the unloaded one. 1 mL of each formulation was prepared and allowed to gel overnight at RT. Initial weights (W_{gel_i}) were recorded for all samples. Subsequently, 1 mL of phosphate buffered saline (pH 7.4, PBS) was gently introduced onto the gels, and the samples were incubated at 37 °C throughout the entire experimental duration. At specific time intervals, samples were collected and weighed after removing excess buffer (W_{gel_f}). At the final time point, all samples were collected, freeze-dried and reweighed ($W_{dried\ gel_f}$). Control samples for hydrogel dissolution assessment were freeze-dried immediately after gel formation ($W_{dried\ gel_i}$). PBS absorption and weight loss were calculated according to the following equations.

$$PBS\ absorption\ (\%) = (W_{gel_f} - W_{gel_i}) / W_{gel_f} * 100$$
$$W\ loss\ (\%) = (W_{dried\ gel_i} - W_{dried\ gel_f}) / W_{dried\ gel_i} * 100$$

E. PLGA NPs release from the hydrogel

The release kinetics of siRNA from the hydrogel loaded with PLGA NPs was tested using PBS as release medium. The test was conducted at 37 °C while shaking the samples at 300 rpm. In order to obtain a release profile, at each time point, the supernatant was collected and replaced with the same amount of fresh buffer. All the supernatants were analyzed by DLS to assess the hydrodynamic diameter and the PDI of the released NPs and at the microplate reader performing the RiboGreen assay to quantify the released siRNA.

F. In vitro testing

The internalization of PLGA NPs by H1299 cells was assessed through an uptake experiment. The cells (30000/ml in 500 µl of medium) were treated 24 hours after seeding with 100 µl of empty PLGA NPs labelled with Coumarin 6 resuspended at different concentrations (i.e., 0.5x, 1x and 2x with respect to the initial solution) either in HPW or in CD solutions at different concentrations (5, 10, 14% w/v).
The PLGA NPs capacity to downregulate protein expression in H1299 cells was evaluated by means of a GFP knockdown test. The H1299/eGFP cells (8000/ml in 500 µl of medium) were treated 24 hours after seeding with 100 µl of PLGA NPs (i) freshly prepared in CD at 14 % w/v, and released from the hydrogel after (ii) 24 and (iii) 48 hours of incubation. All the NPs were prepared employing siGFP. Positive and negative controls were represented respectively by Lipofectamine 2000 lipoplexes with siGFP and PLGA NPs with siNC and siGFP. After 24 hours of incubation for the uptake experiment and 48 hours for the knockdown experiment, cells were analyzed by flow cytometry using 488 nm excitation and 530/30 nm bandpass emission filter set. Median fluorescence intensity (MFI) was obtained analyzing at least 10000 viable cells.

III. Results and Discussion

A. PEU characterization

The PEU synthesis process showed a yield of 78%, and the collected PEU exhibited $\bar{M}_n$ of 24 kDa and polydispersity index of 1.7. The IR spectrum proved the successful PEU synthesis by showing the characteristics peaks of urethane bonds: (i) stretching of N-H bonds at 3348 cm^{-1}, (ii) stretching of C=O bonds at 1721 cm^{-1}, and (iii) simultaneous bending of N-H and stretching of C=O bonds at 1535 cm^{-1}.

B. PLGA NPs characterization

PLGA NPs uptake and tissue penetration are strongly influenced by the size, its distribution and the zeta potential. For this reason, DLS and PALS analyses were performed to evaluate the hydrodynamic diameter, the PDI and the Zeta potential of PLGA NPs. As shown in Fig. 1 (a), the NPs resuspended in CD solution at 14% w/v concentration in HPW exhibited a slight decrease in size with respect to the NPs resuspended in HPW. PDI values were below 0.05, confirming that the NPs containing solution was monodispersed. Both the formulations showed negative zeta potential values. However, the NPs resuspended in CD solution showed a less negative value (Fig. 1 (b)), due to the presence of CDs. Indeed, CD pKa value ranges between 12.1 and 13.5, which leads to an increase of the superficial charge.

The PLGA NPs siRNA encapsulation efficiency was measured to be 55±2 %. This value is coherent with previous studies on this formulation [4].

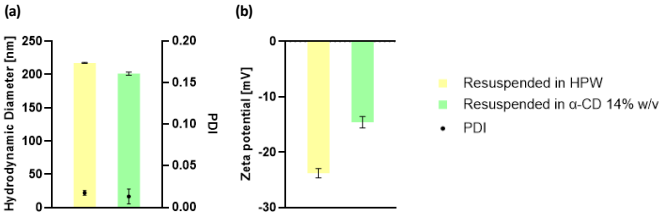

Fig. 1: (a) DLS measurements of PLGA NPs resuspended in HPW (yellow) and in 14% w/v concentrated CD solution (green). Hydrodynamic diameters (left-y axis) and PDI (right y-axis); (b) Zeta potential of PLGA NPs resuspended in HPW (yellow) and in 14% w/v concentrated CD solution (green).

C. Hydrogel characterization

The tube inverting test demonstrated that the PLGA NPs did not affect the hydrogel gelation kinetics as both the NPs loaded and unloaded gels showed a gelation time of around 12 hours. Rheological strain sweep tests (Fig. 2 (a)) indicated that NPs loaded and unloaded formulations exhibited a LVE up to 3 and 7% strain, with complete network disruption at 8% and 50% strain, respectively. The presence of NPs reduced the storage and loss moduli within the LVE, suggesting they acted as defects affecting gel assembly, packing and resistance to applied deformation. Frequency sweep tests (Fig. 2 (b)) confirmed that both formulations maintained a gel-like behavior, with storage modulus (G') consistently exceeding loss modulus (G"). Finally, self-healing strain tests (Fig. 2 (c)) revealed that the loaded formulation showed a slight decrease in recovery capacity after deformation, indicating that the NPs affected the gel self-healing properties.

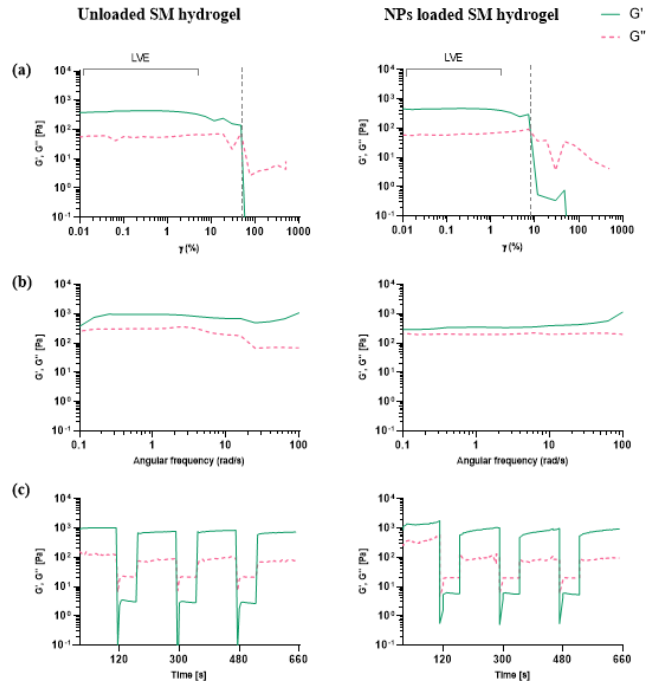

Fig. 2: Strain sweep test (a), frequency sweep test (b) and self-healing test (c) for unloaded (left column) and NPs loaded (right column) SM hydrogel. G' (green) and G'' (pink) are plotted as a function of strain (%) for strain sweep test, of angular frequency for frequency sweep test and time for self healing strain test.

Both PLGA NP-loaded and unloaded SM hydrogels were then subjected to evaluations of their buffer absorption and weight reduction properties in contact with PBS at 37 °C. In Fig. 3 (a), a comparison is made between the swelling behavior of NPs loaded and unloaded hydrogels. Initially, the pristine gels underwent swelling (PBS absorption up to 10% over the initial 8-hour period), followed by a significant reduction in swelling of up to 80%. The presence of PLGA NPs accelerated this reduction in swelling: the hydrogels loaded with PLGA NPs released their components starting from 8 hours of incubation, whereas this phenomenon was observed after 24 hours in unloaded gels.

Concerning the dried weight reduction shown in Fig. 3 (b), the pristine hydrogels experienced an initial decline of 30% of the initial weight during the first three time intervals, culminating in a total loss of 50% of the initial weight after 48 hours of incubation. NP-loaded hydrogels behaved similarly, although they displayed an escalated weight loss trend, ranging from 40% to 60%. This outcome, combined with the observed swelling behavior, validated the findings from prior tests, indicating that the incorporation of PLGA NPs diminished the structural integrity of the hydrogel network.

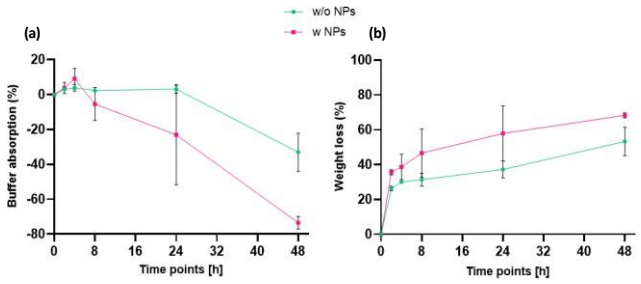

Fig. 3: (a) Buffer absorption (%) measured for SM hydrogel without (green) and with (pink) PLGA NPs at different time points (2h, 4h, 8h, 24h, 48h). (b) Weight loss (%) measured for SM hydrogel formulation without (green) and with (pink) PLGA NPs at different time points (2h, 4h, 8h, 24h, 48h).

D. PLGA NPs release from hydrogel

In order to gain a comprehensive understanding of the NPs release dynamics, multiple investigations were conducted on the samples obtained from the NPs release tests. Both the hydrodynamic diameter and PDI of the released particles, shown in Fig. 4 (a), were significantly higher than the freshly prepared NPs. Moreover, the size was bigger (between 400 and 700 nm) at the initial time points and gradually decreased after 48 hours to finally reach 100 nm. This trend, coupled with the higher PDI values, suggested that during the initial hours, the released particles might have been entangled or mixed with some gel erosion products, which could have been detected by DLS as bigger particles. The quantification of released siRNA in the supernatant was performed using the Ribogreen assay. An initial burst release of siRNA was observed during the first 8 hours of incubation, with complete release occurring after 48 hours of incubation, while approximately half of the siRNA was released after 24 hours (Fig. 4 (b)).

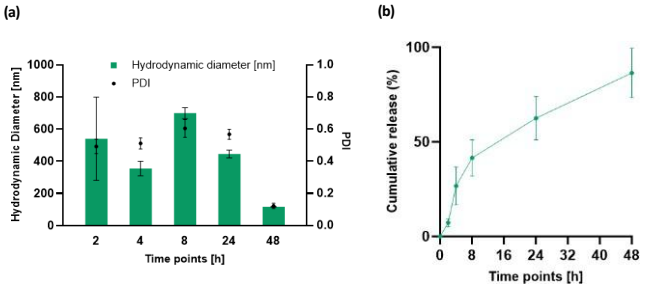

Fig. 4: (a) DLS measurements of the supernatants collected during hydrogel release tests. Hydrodynamic diameters (left y-axis) and PDI (right y-axis) of samples collected at different time points (2h, 4h, 8h, 24h, 48h). (b) siRNA cumulative release (%) over time.

E. In vitro testing

The PLGA NPs uptake capability was tested on the H1299 cell line. As a proof of concept, the test was carried on using only empty and freshly prepared NPs. The test results are shown in Fig. 5 (a). Irrespective of CD concentration, the NPs uptake increased with increasing the NPs concentration. With regards to CD concentration, the addition of CDs at 5% w/v concentration enhanced the uptake efficiency, probably because CDs improved cell membrane permeability. On the other hand, at 10% and the 14% w/v CD concentrations, a decrease in uptake was observed probably due to potential toxic effects. These results suggested that CDs could have a substantial impact on the uptake efficiency of PLGA NPs. Hence, the optimal CD concentration should be carefully determined to balance the permeability effect without inducing excessive toxicity.

After confirming the successful NPs uptake, a gene silencing experiment was performed on the H1299 eGFP cell line. As depicted in Fig. 5 (b), neither freshly prepared PLGA NPs nor the NPs released form the gels exhibited a silencing effect on the cells, as evidenced by the comparison of MFI values measured in cells treated with siGFP and siNC. This outcome can be explained considering the siRNA encapsulation efficiency, which resulted in a realistic content of 25 pmol/well of siRNA. This siRNA amount might not be sufficient to induce a substantial silencing effect. An alternative explanation could involve the occurrence of endosomal entrapment, where the NPs might have been sequestered within endosomes upon their internalization.

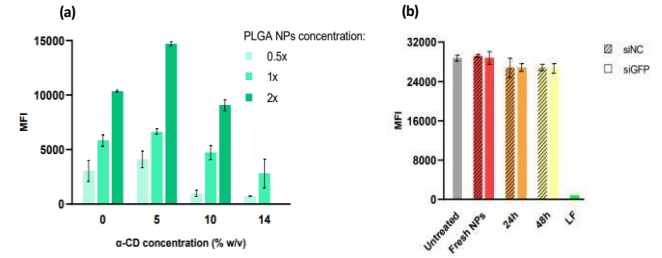

Fig. 5: (a) H1299 uptake of PLGA NPs at three different concentrations with respect to the original solution: 0.5x (light green), 1x (green) and 2x (dark green) in CD solutions at different concentrations (0, 5, 10, 14% w/v). (b) Enhanced green fluorescent protein (eGFP) knockdown efficiency in H1299/eGFP cells after transfection with NPs freshly prepared (red) and released from the hydrogel after 24 (orange) and 48 hours (yellow). The NPs were prepared with both siNC (dashed bars) and siGFP (fully colored bars). The negative and positive controls are represented by untreated cells (grey) and cells treated with Lipofectamine 2000 lipoplexes with siGFP (green).

IV. Conclusion

This research explored the potential of a supramolecular hydrogel system composed of a custom-synthesized PEU and CDs, as an injectable and bioerodible vehicle for localized gene delivery in genetic disorders. Future work should focus on improving gene silencing of released NPs and harnessing biomimetic release mechanisms that allow direct hydrogel interaction with *in vitro* cultures. These insights lay the groundwork for enhancing the hydrogel performance as a localized gene delivery system.

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