

# Supporting information: Pullulan Based Bioconjugates for Ocular Dexamethasone Delivery

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## SI-1 Characterization of pullulan

FT-IR (KBr). 3410 (-OH), 2928 (C-H), 1648 (C-O-C)  $\text{cm}^{-1}$ .

Elemental analysis. Calcd: C, 43.90%; H, 6.28%. Found: C, 40.32%; H, 6.38%.  $^1\text{H}$  NMR (600 MHz,  $\text{D}_2\text{O}$ ).  $\delta$  5.40 (dd, 1H, (1 $\rightarrow$ 4)- $\alpha$ -glycosidic bond), 4.98 (d, 1H, (1 $\rightarrow$ 6)- $\alpha$ -glycosidic bond), 4.09-3.37 (5H, remaining Hs of glucopyranose).

$^{13}\text{C}$  NMR (151 MHz,  $\text{D}_2\text{O}$ ).  $\delta$  100.19, 99.74, 97.89, 95.86, 92.05, 77.74, 77.36, 75.90, 75.65, 74.09, 73.41, 73.31, 73.02, 72.71, 71.68, 71.59, 71.48, 71.39, 71.31, 71.13, 70.78, 70.30, 69.60, 69.55, 69.45, 69.22, 66.46, 63.17, 60.68, 60.54, 60.37.

Data are in accordance with the literature [1–3].

## SI-2 $^1\text{H}$ NMR analysis of pullulan conversion to carboxyethyl-pullulan

Conversion yield of pullulan carboxyethyl-pullulan was measured by  $^1\text{H}$  NMR spectroscopy in deuterated water ( $\text{D}_2\text{O}$ ) according to the general equation (1) [1]:

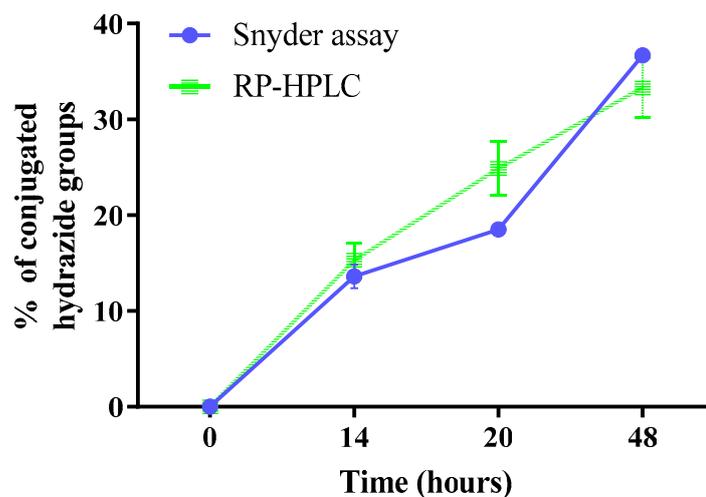
(integral value of selected protons in conjugated group/number of protons of that conjugated group) \*66 (1)

Integration of signal area at 5.45 ppm was set as 1 (1H, (1 $\rightarrow$ 4)- $\alpha$ -glycosidic bond). The integral of signal at 1.33 ppm corresponding to carboxyethyl group (- $\text{CH}_3$ ) was 0.68 which was divided by 3 (3 protons from - $\text{CH}_3$  of carboxyethyl group), multiply by 66 [66 is the number of protons (1H) associated to the (1 $\rightarrow$ 4)- $\alpha$ -glycosidic bond referred to 100 glucose monomers].

In order to calculate the GPU percentage modification with carboxyethyl groups:  $(0.68/3) * 66 = 15\%$  of the GPU modification by carboxyethyl groups in pullulan in the carboxyethyl-pullulan.

## SI-3 Dexamethasone conjugation rate

The dexamethasone conjugation to carboxyhydrazide-pullulan over reaction time was assessed by detecting the disappearance of free dexamethasone by RP-HPLC and hydrazide groups by TNBS. Figure S1 shows the dexamethasone conjugation profile to the polysaccharide. The reaction was terminated when the conjugation yield of dexamethasone was in the 5.0-5.5% GPU range.



**Figure S1.** Kinetic progress of the conjugation between dexamethasone and carboxyhydrazide-pullulan by RP-HPLC analysis (■) and by TNBS assay (●).

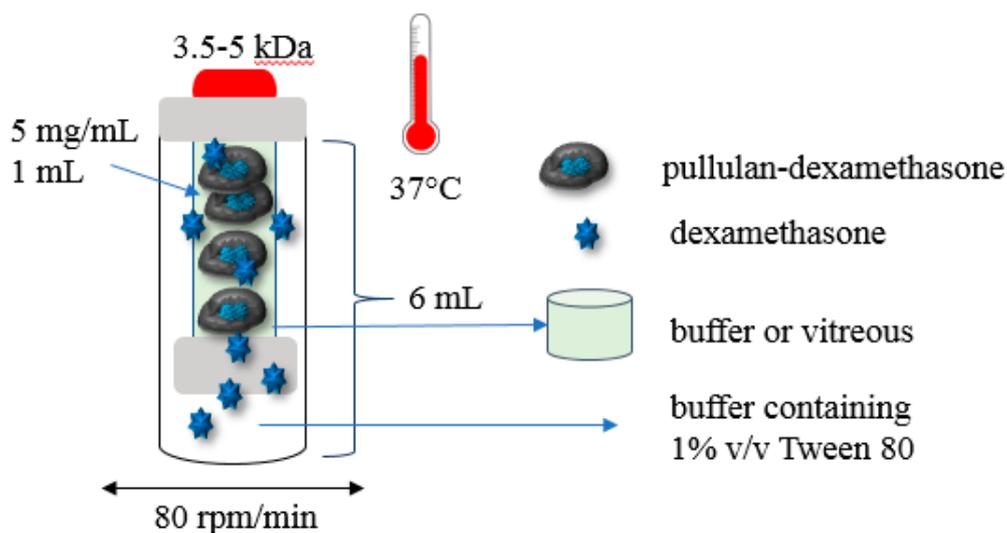
#### SI-4 $^1\text{H}$ NMR analysis of dexamethasone conjugation to carboxyethyl-pullulan

$^1\text{H}$  NMR spectroscopic method was used to derive the pullulan conversion yield in pullulan-dexamethasone, pullulan-dexamethasone-cyanine3 and pullulan-cyanine3. The analysis was performed in  $\text{DMSO-}d_6$  with an internal standard (4-chloro-3-nitrobenzoic acid) and the following equation was then applied:

$$[\text{Integral of signal of internal standard} : \text{Integral of signal of selected protons of sample}] = [\text{number of mmol IS} : \text{number of mmol in selected sample}] \quad (2)$$

The internal standard integrals of signal at known molarity was used to calculate the molarity of dexamethasone in the conjugate pullulan-dexamethasone. Based on the weighed amount of conjugate for the analysis, it was possible to then derive the dexamethasone % w/w in the conjugate and then the GPU units modified with dexamethasone with respect to total GPU.

#### SI-5 Dexamethasone release



**Figure S2.** Schematic representation of the set up for the dexamethasone release by pullulan-dexamethasone under physiologically simulating conditions.

Dexamethasone solubility was assessed in 1% v/v Tween 80 aqueous solution.

A dexamethasone suspension (1.5 mg/mL) was prepared in PBS, pH 7.4, containing 1% v/v Tween 80. The sample was incubated at room temperature with orbital shaking at 100 rpm/min for 20 h in the dark. The suspension was centrifuged at 4000 rpm/min for 20 min and then 1 mL of the supernatant was transferred in a microtube and centrifuged again three times at 14,000 rpm/min for 10 min. A volume of 10  $\mu$ L of supernatant was diluted with 1.49 mL of 0.1 M HCl containing 1% v/v Tween 80. The diluted samples were analysed by RP-HPLC. The solubility of dexamethasone in PBS, pH 7.4, containing 1% v/v Tween 80 was found to be 0.2 mg/mL.

### SI-6 $^{13}\text{C}$ NMR spectra of carboxyethyl-pullulan and carboxyhydrazide-pullulan

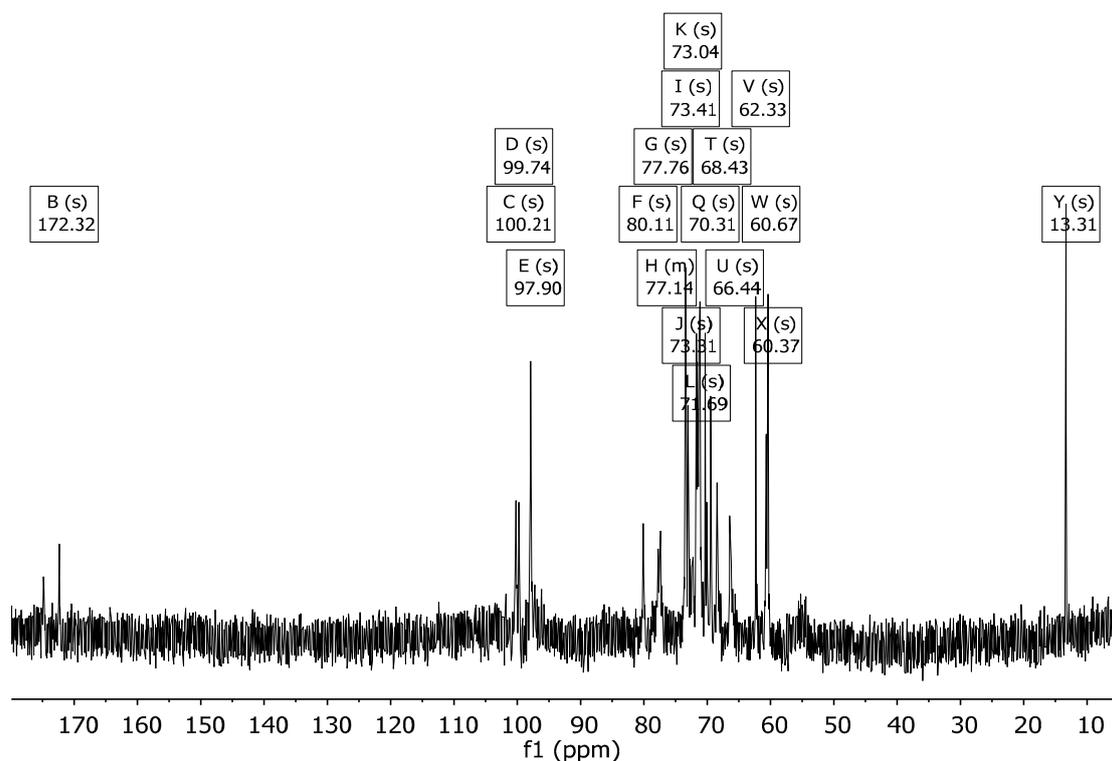
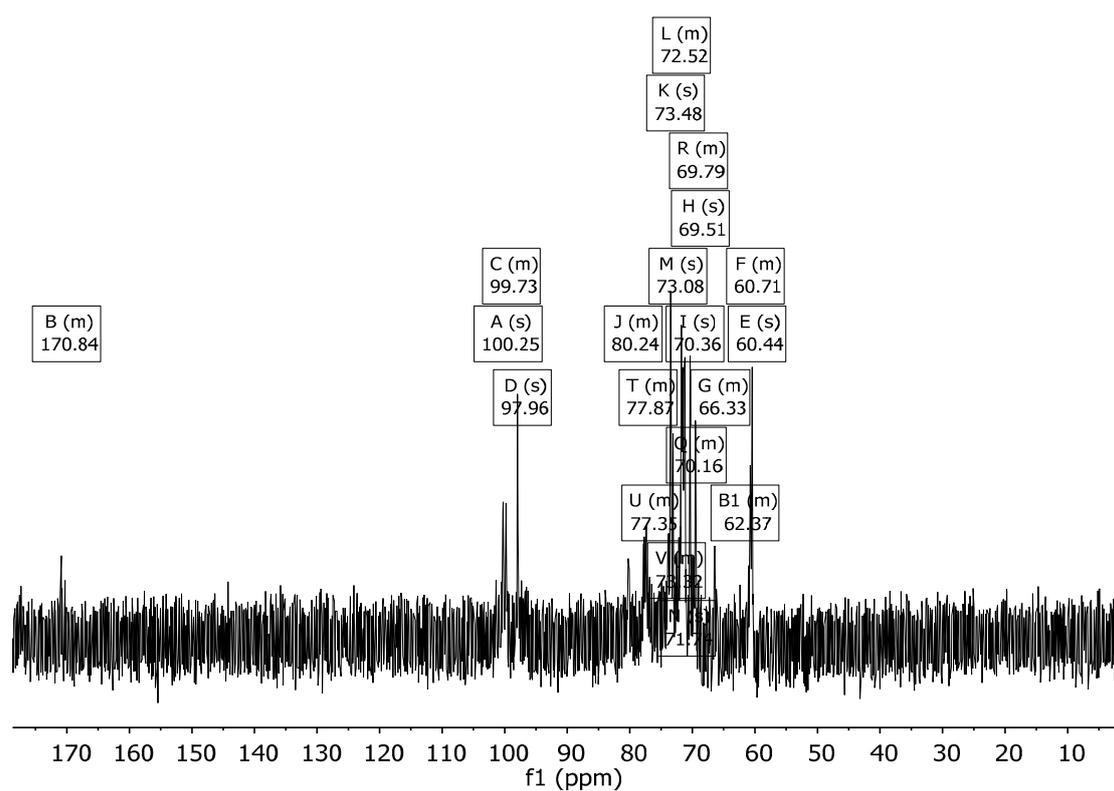


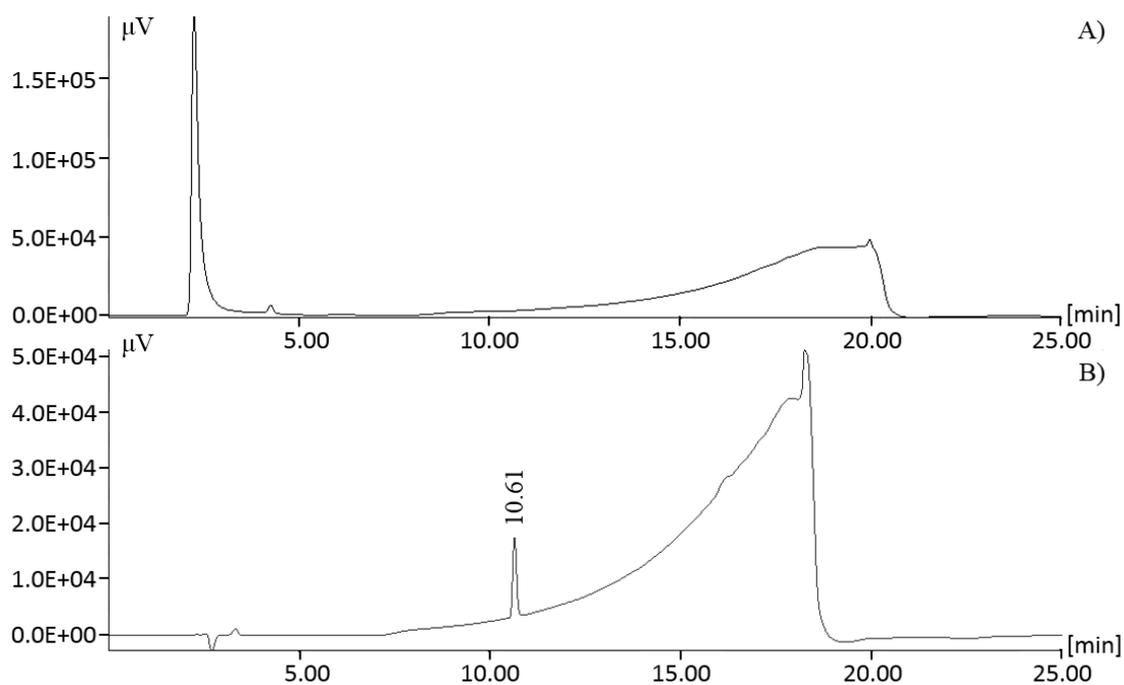
Figure S3. The  $^{13}\text{C}$  NMR spectrum of carboxyethyl-pullulan in  $\text{D}_2\text{O}$ .



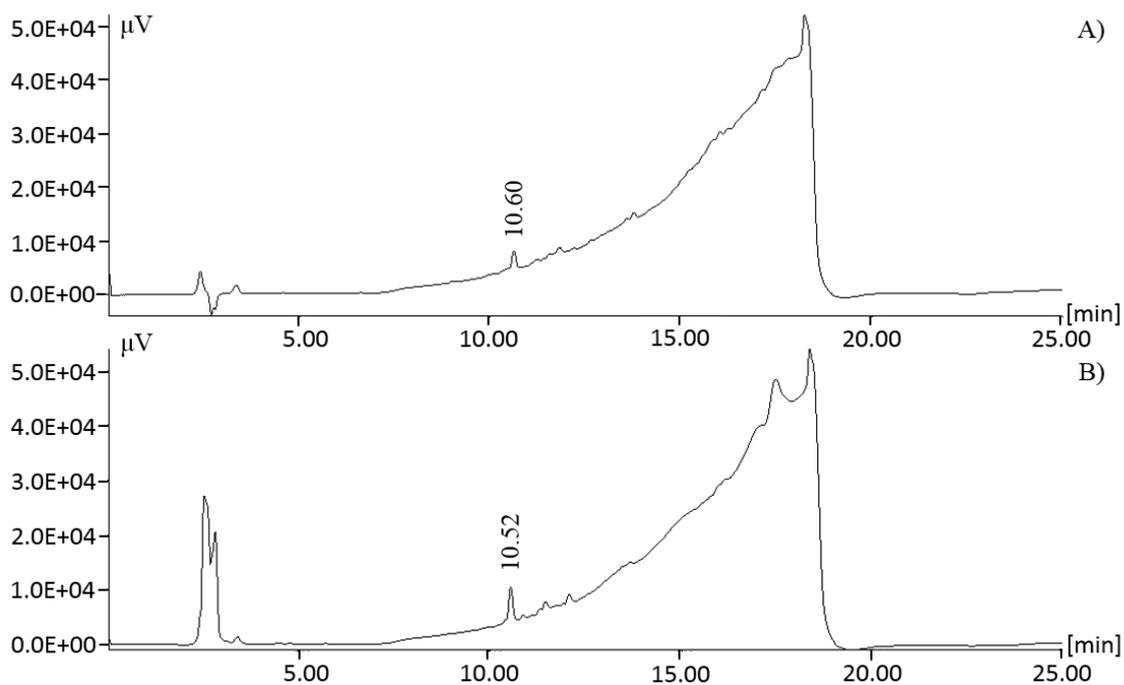
**Figure S4.** The  $^{13}\text{C}$  NMR spectrum of carboxyhydrazide-pullulan in  $\text{D}_2\text{O}$ .

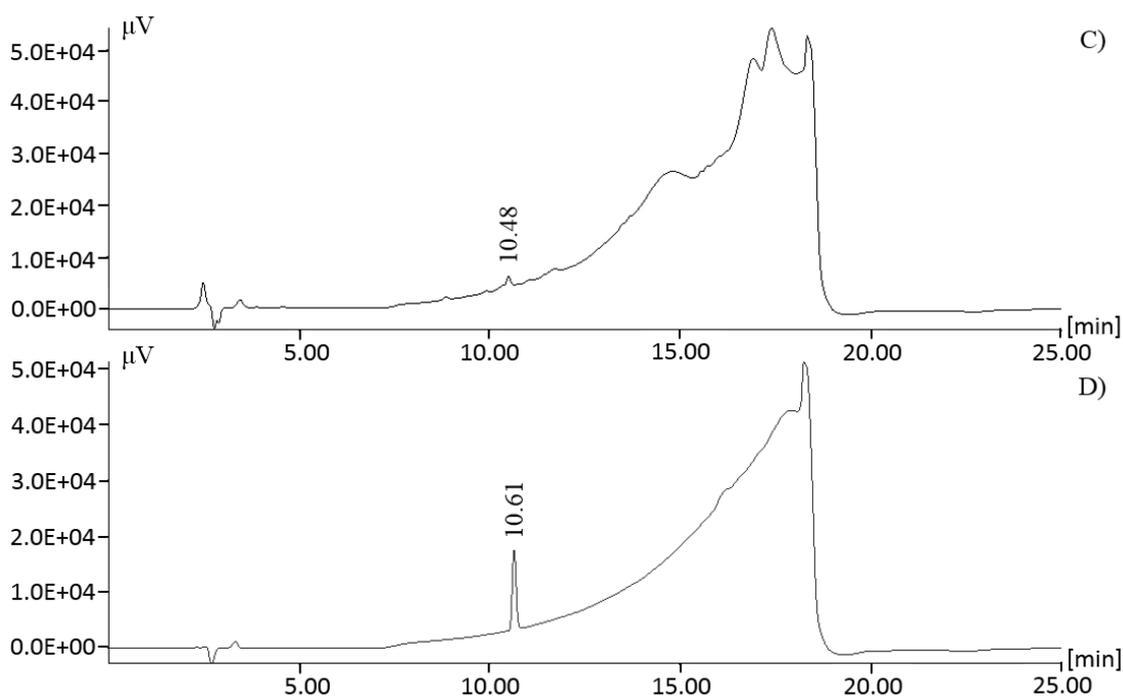
### SI-7 Dexamethasone conjugation

To confirm the efficiency of purification after conjugation of dexamethasone to carboxyhydrazide-pullulan, the presence of free unconjugated dexamethasone in the pullulan-dexamethasone conjugate was assessed with RP-HPLC analysis (Figure S5). The sample was dissolved in PBS, pH 7.4 with 1% v/v Tween 80 (5 mg/mL) and immediately analysed to prevent release of conjugated dexamethasone. No peak of eluted free dexamethasone (retention time = 10.61 min.) was detected on the chromatographic profile. This analysis confirmed the efficiency of the purification method and the purity of the pullulan-dexamethasone.



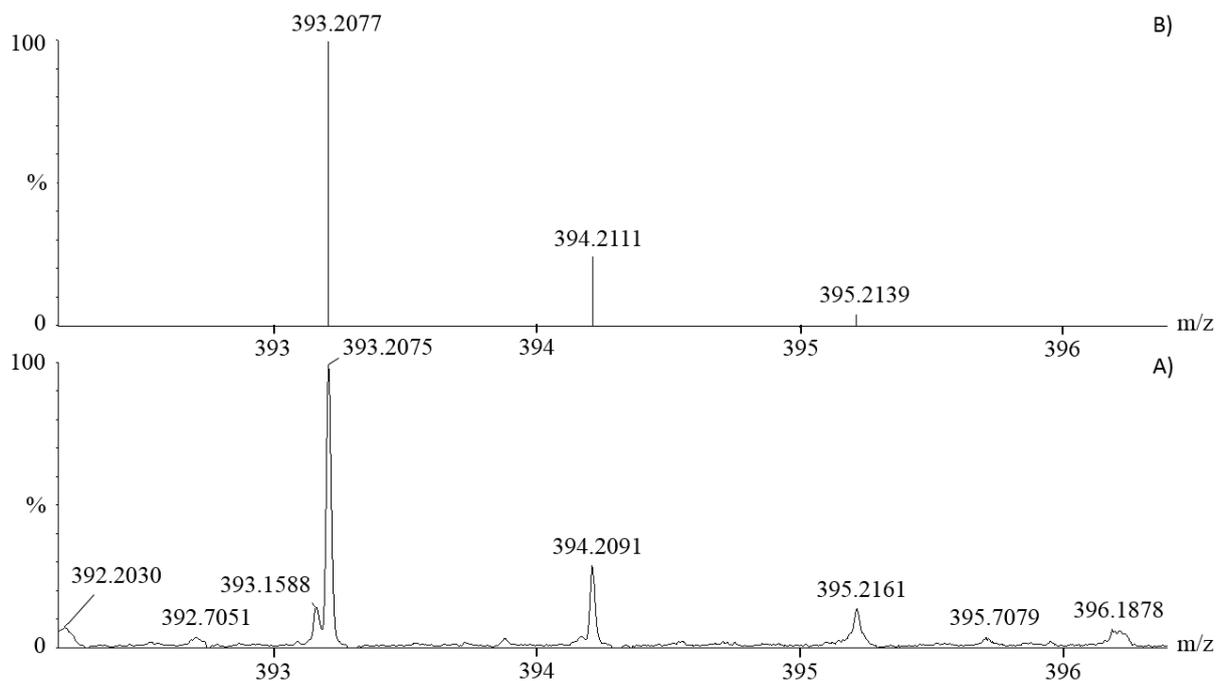
**Figure S5.** RP-HPLC profiles of A) pullulan-dexamethasone after synthesis and purification, and B) free dexamethasone (retention time: 10.61 min).





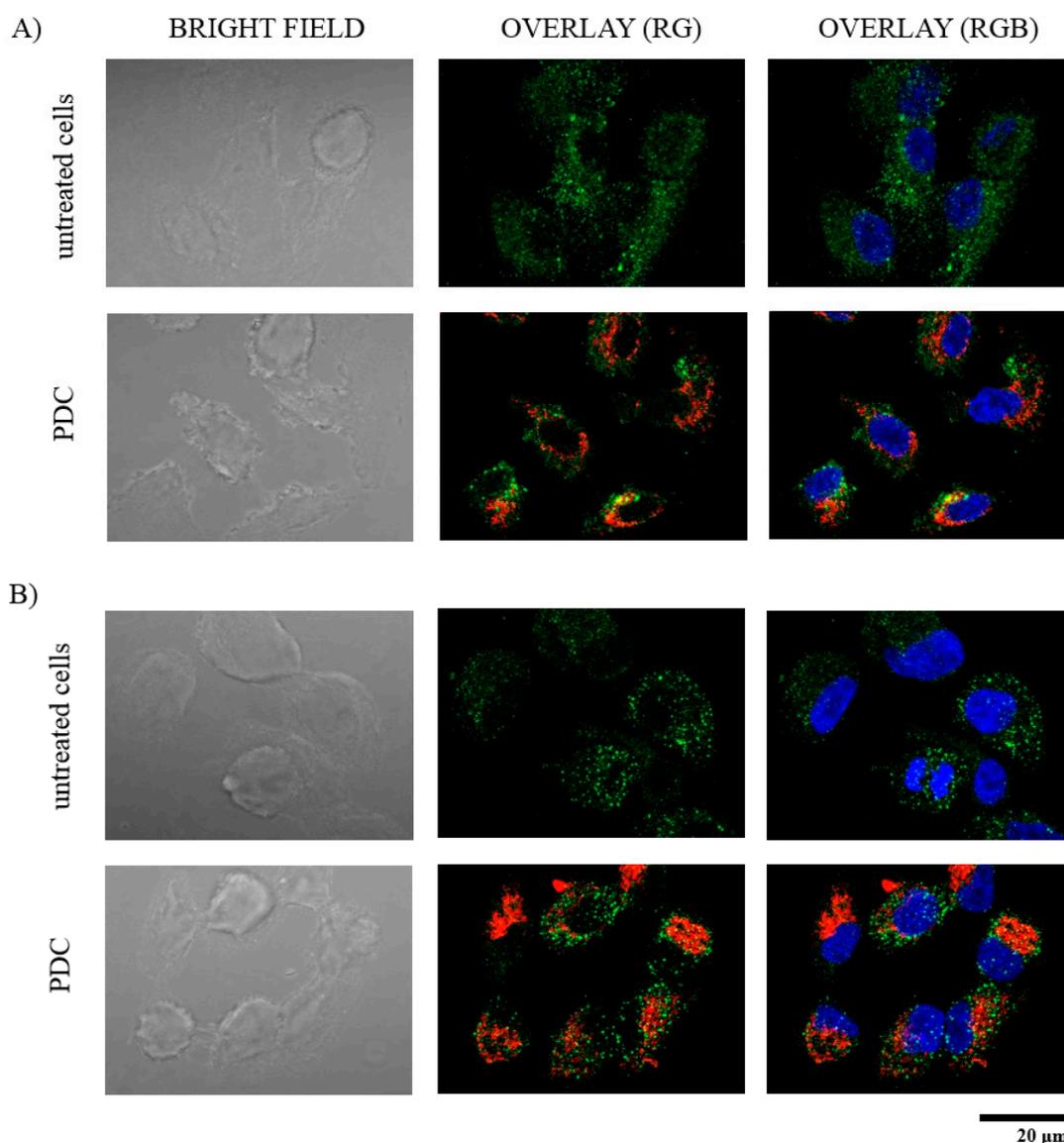
**Figure S6.** RP-HPLC profiles of released dexamethasone from pullulan-dexamethasone in different pH conditions: A) PBS, pH 7.4; B) 10 mM phosphate, 24 mM citric acid, 150 mM NaCl, pH 5; C) 1:1 v/v homogenized vitreous:PBS, pH 7.4; D) free dexamethasone in PBS, pH 7.4. The chromatographic profiles correspond to samples analyzed after 4 days of release.

The experimental spectrum of the released dexamethasone (Figure S7A) in positive ionization shows a  $m/z$  of 393.2075 ( $[M+H]^+$ ) which is in agreement with the expected mass spectrum in positive ionization ( $m/z$  calculated 393.2077) of dexamethasone (Figure S7B).



**Figure S7.** ESI-ToF mass spectrum profiles of dexamethasone in positive ionization: A) experimental mass spectrum; B) expected mass spectrum. The experimental mass spectrum was performed with drug released by pullulan-dexamethasone after 19 days.

## SI-8 Confocal microscopy



**Figure S8.** Confocal microscopic images of ARPE-19 cells incubated with 0.2 mg/mL of pullulan-dexamethasone-cyanine3 (PDC) nanoparticles in red with bright fields. (A) Endosomes were stained in green with rabbit anti-EEA1 and Alexa Fluor 488 labeled goat anti-rabbit IgG H&L secondary antibody. (B) Lysosomes were stained in green with rat anti-mouse anti-LAMP1 and Alexa Fluor 488 labeled goat anti-rat IgG H&L secondary antibody. Nuclei were stained with DAPI in blue. RG: overlay of red and green channels and RGB: overlay of red, green and blue channels for organelles/nanoparticles colocalization. Bar size: 20  $\mu\text{m}$ .

## References

- [1] F. Hassanzadeh, J. Varshosaz, G.A. Khodarahmi, M. Rostami, F. Hassanzadeh, Biotin-encoded pullulan-retinoic acid engineered nanomicelles: preparation, optimization and in vitro cytotoxicity assessment in MCF-7 cells, *Indian J. Pharm. Sci.* 78 (2016) 557–565.
- [2] T.D. Leathers, Biotechnological production and applications of pullulan, *Appl. Microbiol. Biotechnol.* 62 (2003) 468–473.
- [3] N. Tamura, M. Hirota, T. Saito, A. Isogai, Oxidation of curdlan and other polysaccharides by 4-acetamide-TEMPO/NaClO/NaClO<sub>2</sub> under acid conditions, *Carbohydr. Polym.* 81 (2010) 592–598.
- [4] S.L. Snyder, P.Z. Sobocinski, An improved 2, 4, 6-trinitrobenzenesulfonic acid method for the determination of amines, *Anal. Biochem.* 64 (1975) 284–288.