Multifunctional stealth chitosan nanogels with improved relaxivity and photoacoustic contrast for dual MRI–MSOT imaging.

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Supplementary information

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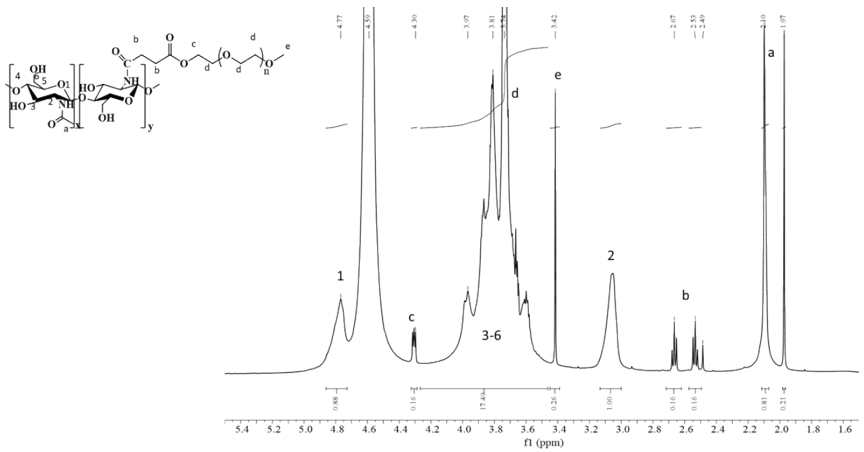

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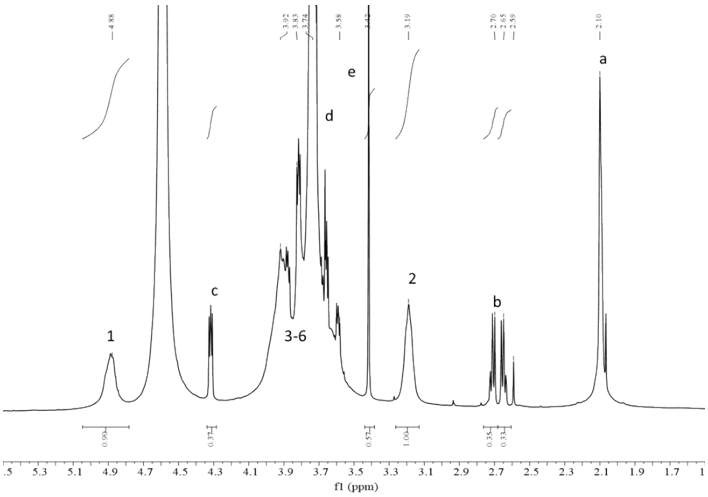

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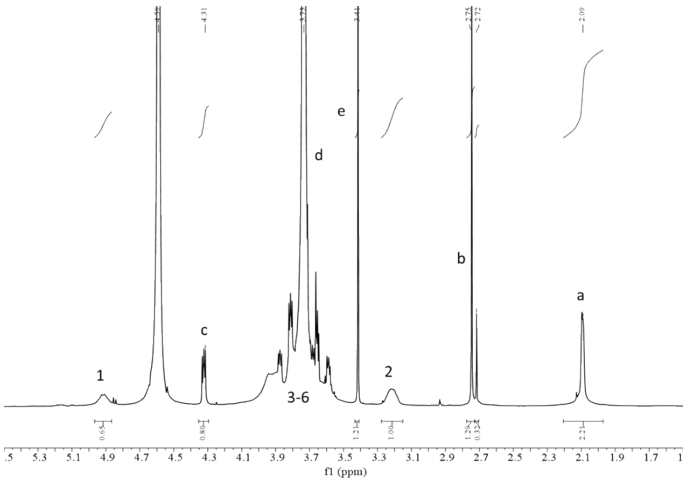


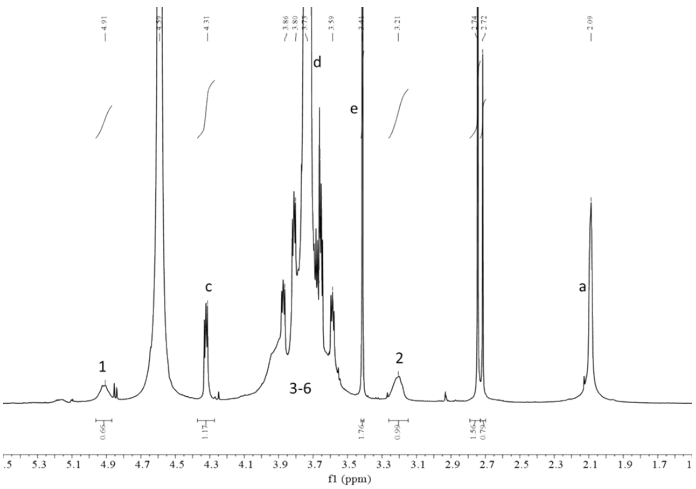
d	

Figure S1: ^{1}H NMR spectra of CS-PEG_{2000} copolymers with (PEG/CS) initial ratios (expressed as % mol $(COOH/NH_2)_{initial}$) of a- 10%, b- 20%, c- 35%, d- 50% (500 MHz, D_2O/DCl 700/1 v/v, 398K)

Determination of DS_{CS}^{PEG}:

The diffusion curves were extracted from CS-mPEG$_{2000}$ DOSY spectra for the more intense peak of PEG at 3.7 ppm and were characterized by two contributions: one coming from the ungrafted PEG (PEG$_{UG}$) which diffuses fast (diffusion coefficient D_{UG}), and the other coming from the grafted PEG (PEG$_G$, diffusion coefficient D_G). Diffusion curves can thus be fitted with a bi-exponential equation according to the procedure developed in ref [15a]. Assuming that CS-PEG$_{2000}$ molecular weight must be close to the one of CS (due to the large difference between PEG$_{2000}$ and CS molecular weights) one can consider that grafted PEG$_{2000}$ (and then CS-PEG$_{2000}$) has the same diffusion coefficient as CS. During the fitting, D_G and D_{UG} were then fixed to values measured independently on chitosan and PEG, respectively: $D_{CS} = 5\times10^{-12}$ m^2.s^{-1}, $D_{PEG2000} = 1.4\times10^{-10}$ m^2.s^{-1}.

The values of I_G and I_{UG} extracted from the fitting allowed to calculate the percentage of grafted PEG over the total amount of PEG (PEG_G/PEG_T):

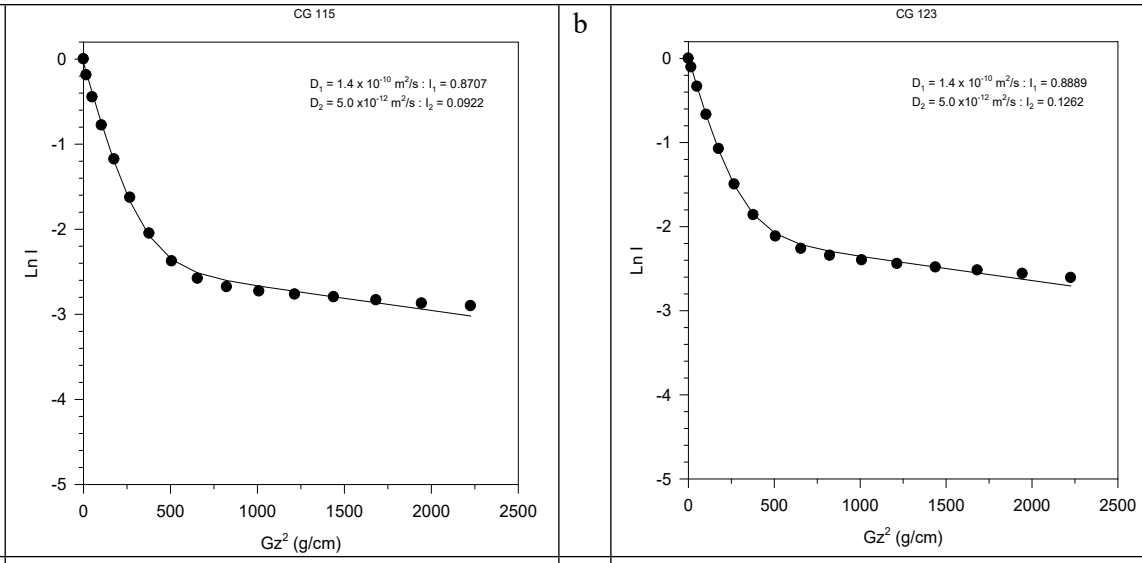
$$\frac{PEG_G}{PEG_T} = \frac{I_G}{I_G + I_{UG}} \times 100 \qquad \text{eq 1}$$

The percentage of PEG$_{2000}$ grafted to CS chains ($DS_{PEG/CS}$) was then calculated from ^{1}H NMR and DOSY experiments:

$$DS_{PEG/CS} = \%\frac{PEG_G}{CS} = \frac{[PEG]_t}{[CS]} \times \frac{PEG_G}{PEG_T} = \frac{(I_{H_b} + I_{H_{b'}} + I_{H_c} + I_{H_e})/9}{(I_{H_1} + I_{H_2})/2} \times 100 \times \frac{I_G}{I_G + I_{UG}} \times 10$$

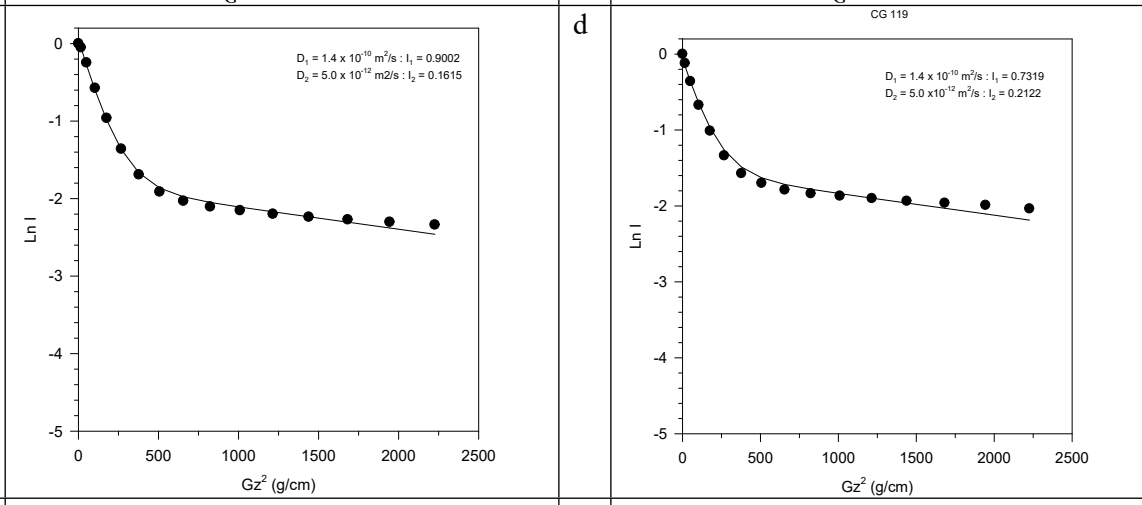
eq 2

where I represented the integration of the peaks indicated in brackets, whereas I_G and I_{UG} standing for the intensities extracted from the DOSY experiments, for grafted and ungrafted PEG respectively.



$I_{UG} = 0.8707$
$I_G = 0.0922$

$I_{UG} = 0.8889$
$I_G = 0.1262$



$I_{UG} = 0.9002$
$I_G = 0.1615$

$I_{UG} = 0.7319$
$I_G = 0.2122$

Figure S2: Diffusion curves and intensities I_{UG} and I_G extracted from DOSY spectra of CS-PEG_{2000} copolymers (UG corresponds to ungrafted PEG chains, $D_{UG} = 1.40 \times 10^{-10}$ m^2 s^{-1} while G corresponds to grafted PEG chains, $D_G = 5 \times 10^{-12}$ m^2 s^{-1})

Table S3: Determination of CS-PEG_{2000} degree of substitution DS_{CS}^{PEG} according to (PEG/CS) initial ratios (expressed as % mol $(COOH/NH_2)_{initial}$)

%mol $(COOH/NH_2)$initial	PEG_T / CS (%) RMN ^1H	PEG_G / PEG_T (%) DOSY	DS_{CS}^{PEG} = PEG_G / CS (%)
10	8.2	9.6	0.8
20	18.0	12.4	2.2
35	35.4	15.2	5.4
50	46.7	22.5	10.5

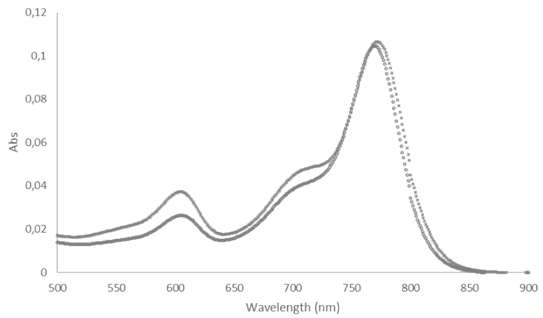
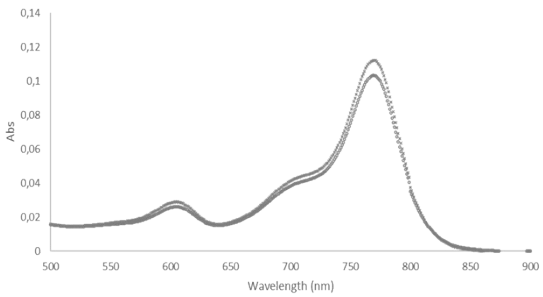
a-	b-
	

Figure S4: UV-Visible spectra of a- (CS-PEG$_{2000}$/CS-ZW800)/TPP and b- GdDOTA⊂(CS-PEG$_{2000}$/CS-ZW800)/TPP nanogels before (×) and after (🐟) purification

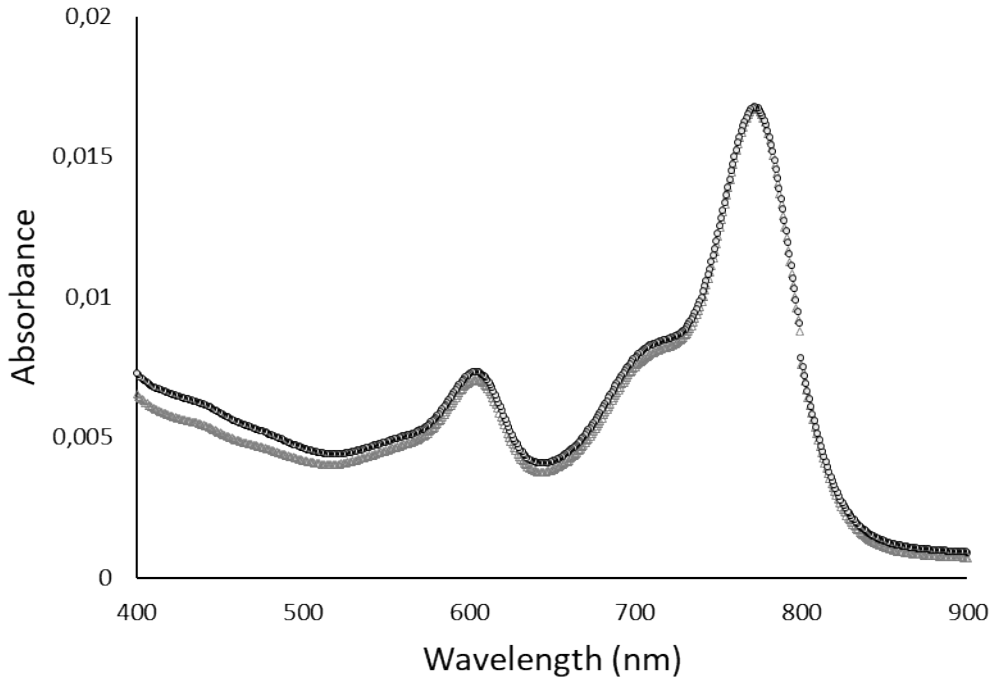


Figure S5: UV-Visible spectra of GdDOTA⊂(CS-PEG2000/CS-ZW800)/TPP (🐟) and (CS-PEG2000/CS-ZW800)/TPP (❐) nanogels after purification ([ZW800] = 5µM)

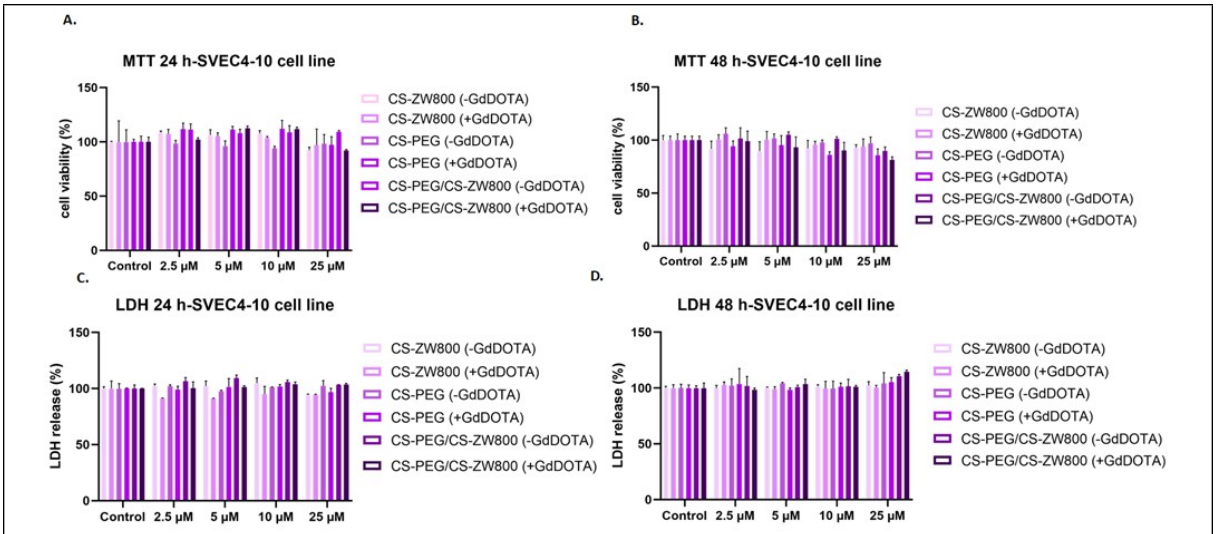


Figure S6: Cell viability and cytotoxicity of SVEC4-10 endothelial cells after exposure to various concentrations of nanogels (NGs). (A, B) Cell viability assessed by MTT assay after 24 h (A) and 48 h (B) of treatment. (C, D) Cytotoxicity evaluated by lactate dehydrogenase (LDH) release in the culture medium after 24 h (C) and 48 h (D) of exposure. Results are expressed as percentages relative to untreated controls.

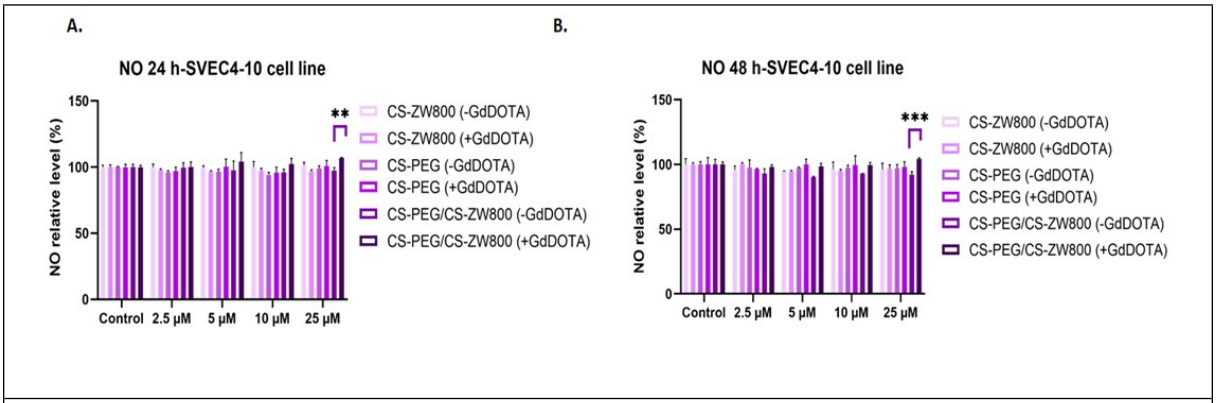


Figure S7: Nitric oxide (NO) production by SVEC4-10 endothelial cells after exposure to nanogels (NGs). NO levels were measured in the culture medium using the Griess assay after 24 h (A) and 48 h (B) of treatment with increasing NG concentrations. Results are expressed as percentages relative to untreated controls (set as 100%) and are presented as mean ± SD (n = 3). Statistical significance was determined by two-way ANOVA followed by Tukey's multiple comparisons test: **$p < 0.01$**, ***$p < 0.001$ ***.

S8: Gd loading determination

To determine their Gd content, the nanogels were mineralized in aqua regia, a medium that is not only more acidic than 1 M HCl but also strongly oxidizing.
To verify that these conditions efficiently release Gd ions from both the GdDOTA complex and the nanogel matrix, the evolution of the longitudinal relaxation rate (R_1) of GdDOTA(CS-PEG2000/CS-ZW800)/TPP nanogels in aqua regia was monitored over 24 hours. Under these conditions, an increase in R_1 is expected, as the coordination sphere of the Gd^{3+} ion should evolve toward that of the fully hydrated aqua complex, $Gd(H_2O)_{8-9}$.

As shown in Figure S8 (measured at 40 MHz), R_1 increases rapidly from an initial value of 7.41 s^{-1} and reaches a plateau after approximately 3 hours, with a maximum value of 12.99 s^{-1}. This value is close to the R_1 measured at the same concentration for $Gd_2(SO_4)_3$ (11.36 s^{-1} at 40 MHz), incubated with a nanosuspension of Gd-free nanogels and subsequently mineralized in aqua regia.

These results indicate that, in aqua regia, both the nanogel matrix and the Gd chelates are fully degraded. The released Gd^{3+} ions adopt a coordination environment comparable to that in the $Gd_2(SO_4)_3$ salt, namely a single Gd^{3+} ion surrounded by 8 to 9 water molecules.

Similar results were obtained for GdDOTA(CS-PEG2000)/TPP nanogels.

Finally, after the mineralization step, the solutions were introduced into an inductively coupled plasma operating at temperatures above 7,000 K. The prior mineralization in aqua regia ensures complete digestion of the samples and results in solutions with uniform viscosity, thereby minimizing transport-related interferences and variability in the volume of solution introduced into the ICP-OES instrument.

These controlled experimental conditions reduce potential analytical biases and improve the accuracy and reliability of gadolinium quantification in the nanogels.

S9: Procedure used to determine the relaxivity r_1 of GdDOTA(CS-PEG2000)/TPP and GdDOTA(CS-PEG2000/CS-ZW800)/TPP nanogels

The procedure used to determine the paramagnetic r_1 relaxivity of the nanosuspension according to the proton Larmor frequency is the following:

1. The longitudinal relaxation time from either GdDOTA(CS-PEG_{2000})/TPP or GdDOTA(CS-PEG_{2000}/CS-ZW800)/TPP nanogels was recorded according to the proton Larmor frequency ($T_{1\ \text{observed}}$)
2. Diamagnetic contribution was measured by recording longitudinal relaxation time from unloaded nanoparticles at the same NP concentrations than Gd-loaded samples ($T_{i\ \text{dia}}$).
3. The paramagnetic relaxation rate R_{1para} was calculated according to: $R_{1para} = (1/T_{1\text{para}}) = (1/T_{1\text{observed}}) - (1/T_{i\ \text{dia}})$

The relaxivity r_1 was then obtained according to: $r_1 = \frac{R_{1para}}{[Gd]}$ where [Gd] is determined by ICP-OES for each Gd-containing nanosuspension.