G. Ronzoni Institute

GELATIN FUNCTIONALIZATION WITH SULFATED GLYCOSAMINOGLICANS

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INTRODUCTION

Biomaterials for biomedical and medical devices applications are composed by natural, synthetic or hybrid reticulated polymers and they can also be functionalized with active molecules, through bio-conjugations, to obtain suitable structural properties such as three-dimensional scaffolds or hydrogels that induce the formation of tissues and provide tissue models in vitro to study cellular responses in an environment more similar to biological system. [1]-[2]

Gelatin is a biopolymer obtained from the acid or alkaline hydrolysis of collagen, one of the most abundant proteins in mammals (about 30%). It has a chemical and amino acid composition similar to that of collagen, but a less "ordered" macromolecular structure. [3] Gelatin is biodegradable, biocompatible, relatively inexpensive. All these features make it ideal for the design and development of new functional materials for biomedical applications. In addition, gelatin-based materials are excellent bioactive compounds to support cell adhesion and proliferation, thanks to the presence of correct biological signal. [4]

Heparin is a sulfated linear polysaccharide consisting of repetitive disaccharide sequences composed of uronic acid linked to D-glucosamine (GlcN), present mainly in the N-sulfated form, and which carry O-sulfated groups in positions 2 and 6 and belongs to the Glycosaminoglycan family. [5] It is one of the main pharmaceutical agents used in the treatment and prevention of blood clots or thrombi and is generally obtained from the intestinal mucosa or other usable organs of some slaughter animals. [6]

AIM

- To functionalize the gelatin with Heparin to create a possible scaffold which can be used for biomedical applications. [7]

Strategy steps:

- Synthesis of the functionalized gelatin
- Characterization through NMR, GPC-SEC, DLS and Rheometer measurements of the derivates and starting materials.
- Study of their interaction with physiological proteins [8]

RESULTS

Synthesis of functionalized gelatin :

The first step was the partial deamination of unfractionated heparin, in order to afford un aldehyde terminal (**1**), which reaction is illustrated in Fig. 1.

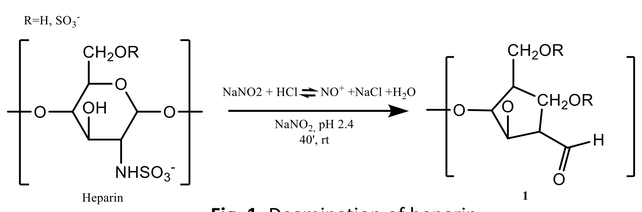


Fig. 1. Deamination of heparin.

The deaminated heparin was then conjugated to the gelatin, to obtain final compound **2**, Fig. 2. One-step reductive amination reaction has been performed using different equivalents of NaBH3CN and $NaBH_4$ as reductive agents. It was observed that the best conditions are in presence of 0,6 equivalent in weight of $NaBH_3CN$. Compound 2 was then purified with dialysis (c/o 12000-14000) in water.

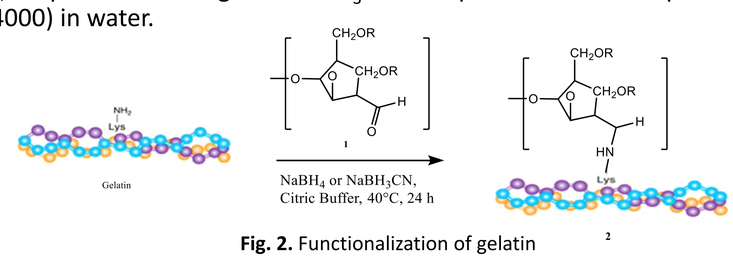
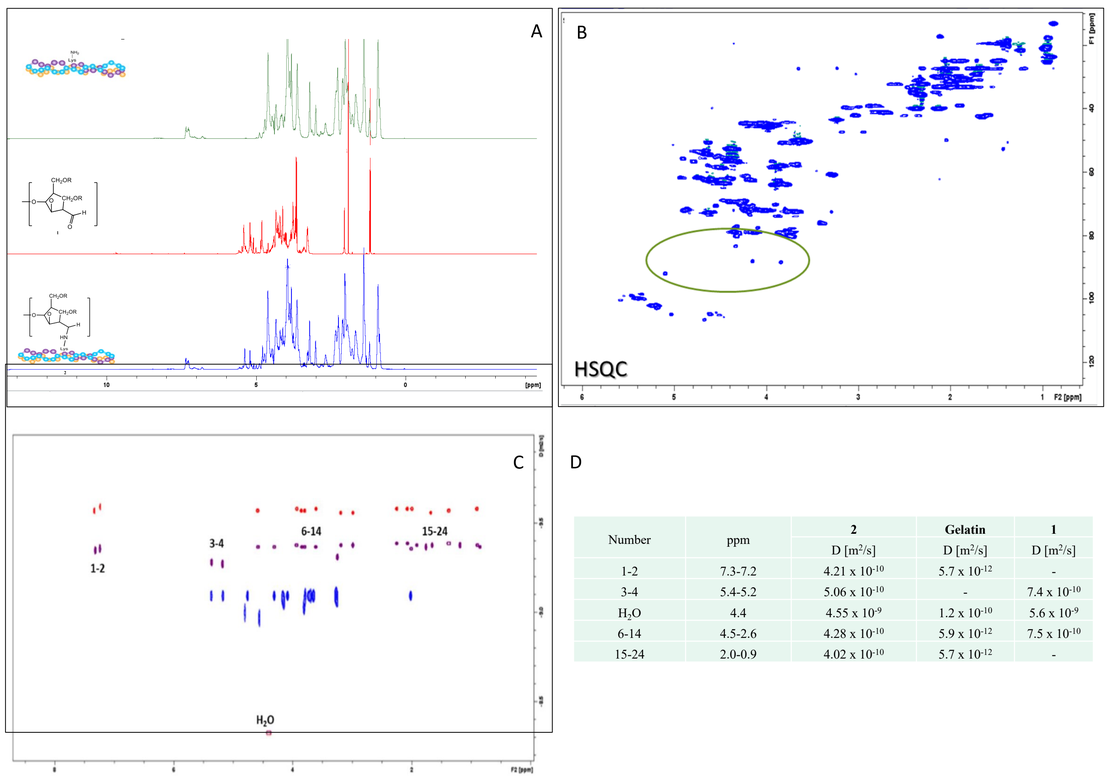


Fig. 2. Functionalization of gelatin

Caracterization of the compounds:

The final compound **2** has been characterize through NMR, in particular with ^1H NMR and HSQC, Fig. 3. From HSQC, Fig. 3B, is possible to observe the presence of signals between 4.5 and 5.5 ppm which have been attributed to the heparin structure.



Number	ppm	2	Gelatin	1
		D [m²/s]	D [m²/s]	D [m²/s]
1-2	7.3-7.2	4.21×10^{-10}	5.7×10^{-12}	-
3-4	5.4-5.2	5.06×10^{-10}	-	7.4×10^{-10}
H_2O	4.4	4.55×10^{-9}	1.2×10^{-10}	5.6×10^{-9}
6-14	4.5-2.6	4.28×10^{-10}	5.9×10^{-12}	7.5×10^{-10}
15-24	2.0-0.9	4.02×10^{-10}	5.7×10^{-12}	-

Fig. 3. All the NMR spectra were acquired using Bruker avance HD NMR instrument 500 MHz, equipped with probe TCI, 5 mm at 70° C. **A)** ^1H-NMR of Gelatin (in green), Deaminated Heparin **1** (in red), and conjugated compound **2** (in blue). **B**) HSQC of Compound 2, circled in green the signals attributed to the heparin linked to the gelatin. **C**) DOSY spectra of deaminated Heparin **1** (in blue), Gelatin (in red) and Compounds **2** (in purple). **D**) Diffusion coefficient (D) of the three different compounds (**1**, **2** and Gelatin).

However due to the articulated nature of the gelatin, the identification of the boundary peaks in the NMR spectra is complicated. To confirm the conjugation a DOSY experiment was done, in order to differentiates species in mixture according to the molecular weight. DOSY Spectra and Diffusion coefficient are reported in Fig 3C and table 3D. It has been possible to confirm that heparin is chemically linked to the gelatin, for the presence of a single diffusion coefficient for all the signals of the proton spectrum. Moreover, Final compound **2** present a higher Diffusion coefficient than the one of the heparin, but lower than the one of the gelatin.

The molecular weight of the compounds synthetized have been obtained by Gel permeation techniques size exclusion chromatography (GPC-SEC). Results in terms of Average molecular weights (Mw), Number average molecular weight (Mn), Distribution (Mn/Mw), Hydrodynamic ratio (Rh), viscosity (η) and recovery in %, are reported in Tab. 1. It is possible to observe an increase of the molecular weight in the conjugated compound 2, which present a Mw of 232 kDa, compared to the starting gelatin (Mw of 171 kDa).

Sample	Mn (KDa)	Mw (KDa)	Mn/Mw	Rh (nm)	[η] (dl/g)	% Recovery
Heparin	12,8	18,2	1,4	3,9	0,2	99
Gelatin	78,5	171,7	2,2	9,2	0,3	98
1	4,8	6.8	1,4	2,0	0,1	77
2	78,0	232	2,9	9,0	0,3	84

Tab. 1. GPC-SEC-TDA results of heparin, gelatin, deaminated heparin (1) and final compound 2. Obtained with Malvern instrument, column system: TSKGel GMPWXL, temperature: 40° C, Mobile phase: 1 M K_2HPO_4+ 0,006 M KH_2PO_4/ $NaNO_3$ 0,125 M at pH 6.7. The Dn/Dc has been calculated for compounds **2** and it is equal to 0.132 ml/g, while for Gelatin and Heparin were used the corrispondents values of 0.163 and 0.13 reported in literature. [9]

Sample	Zp (mV)
Gelatin	-2
1	-36
2	-19

Tab. 2. Zeta Potential results obtained with Malvern Zetasizer instrument, of gelatin, deaminated heparin, and conjugated compound and dissolved in HBSS at 5%

In Tab.2 is reported the Zeta potential (Zp) of the gelatin, deaminated heparin and compound **2.** It is possible to observe that for gelatine this value is, at neutral pH -2, according to its chemical structure indicating a surface charge near to zero. While for the deaminated heparin is extremally negative (Zp is close to -40 mV) consistent with its anionic nature due to the carboxyl and sulfate groups. Finally, the gelatin-heparin compound shows a negative Zp, of about -19, index of the derivatization of the gelatin with heparin.

The compound synthetize has been studied also from the rheological point of view, Fig. 4. It is possible to observe that the viscosity of the final conjugate **2**, in blue, exhibits a fluidifying behavior when sheared, characterized by a decrease in viscosity as the cutting speed increases, such as for gelatin, reported in green. However, Compound **2** presents in absolute terms a lower viscosity than gelatin, in accordance with the result of the HP-SEC-TDA analysis.

Compound **2** has been also compared to a physical mixture of gelatin and Clexane, a commercial low molecular weight heparin, more similar for its structure to the deaminated heparin. The results show that gelatin and Clexane flow curve maintains the same viscosity curve as the gelatin, providing another confirmation of the chemical bond between heparin and gelatin, which modifies the viscosity of the product. [10]

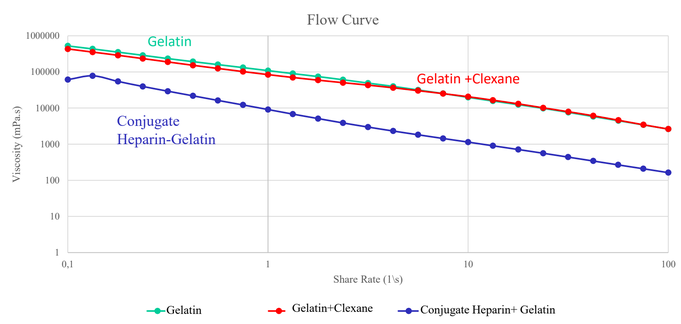


Fig. 4. All the flow curves have been obtained using MCR 92 Anton Parr instruments, equipped with a plate plate geometry, all the measures have been acquired at 25° C , in the range of 0,1 to 1000 1/s. In the figure are reported the flow curves of Gelatin (in green), Gelatin and Clexane (In red) and of compound 2 (in blue).

Study of the interaction with proteins:

In order to evaluate whether the heparin linked to gelatin maintains its biological activity, the interaction with protamine [11] and Plate Factor 4 (PF4) [12] have been investigated, through Zp studies Fig. 5. To demonstrate this interaction, the Zp values were measured as a function of the protein/ polymer ratio (PHR). If heparin maintains its biological activity of binding positively charged proteins, it must be able to screen the positive charges of protamine and PF4 and thus achieve neutrality. [8]

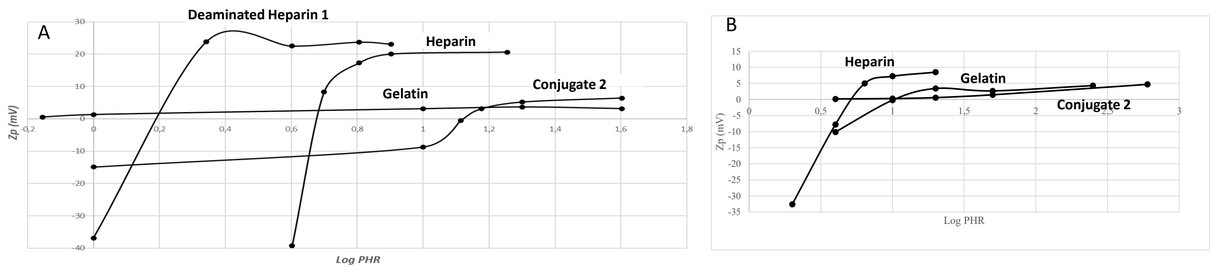


Fig. 5. Zp results express in term of log PHR vrs Zp for A) PF4 and B) protamine. These results were obtained using Zetasizer Malvern Instrument. Zp results have been measured for all the samples tested in function of the protein/polymer ratio.

In the figure is possible to observe that the point where the curve reaches neutrality: towards a PHR of 13 for protamine and between PHR 10-20 for PF4. This is a very good result as it means that little sample is required to neutralize protamine or PF4.

CONCLUSIONS

The synthesis procedure adopted gave a positive result, allowing to obtain a gelatin derivatized with a heparin, and the sinthetic strategy has been improved.

Starting materials and final products have been characterize by NMR, GPC-SEC-TDA, Zeta potential, and viscosity measures. The analisys confirmed the presence of a chemical bond between heparin and gelatin, which influence the features of the final compounds, such as the viscosity, Zeta potenzial and molecular weight.

Finally, it has been observed, by proteins interaction studies, that heparin maintains an interaction with PF4 and protamine even after being chemically linked to gelatin, which in any case has an active role in this type of interaction.

Future developments will include the cross-linking of the final product in order to obtain hydrogels or new biocompatible functionalized materials.

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