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## Exploring the use of natural zeolite to engineer green therapeutic agents for the advanced treatment of chronic wounds

**Rossella Laurano<sup>1</sup>, Jacopo Secco<sup>2</sup>, Hans Wiech<sup>3</sup>, Gianluca Ciardelli<sup>1</sup>**

<sup>1</sup>*Department of Mechanical and Aerospace Engineering, Politecnico di Torino, Corso Duca degli Abruzzi 24, Italy*

<sup>2</sup>*Department of Electronics and Telecommunications, Politecnico di Torino, Corso Duca degli Abruzzi 24, Italy*

<sup>3</sup>*AURANICUM holistic health UG, Jarnostr. 17, D-22145 Hamburg, Germany*

*Corresponding authors: Rossella Laurano, +39 3405561248, rossella.laurano@polito.it*

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### Introduction

Chronic skin wounds are defined as lesions that fail to heal within 3-4 weeks of treatment, primarily due to persistent inflammation and infection. Despite great efforts have been devoted towards the engineering of anti-inflammatory and anti-bacterial formulations, the effective management of chronic wounds remains a substantial clinical challenge. Consequently, the pursuit of innovative and more efficacious therapeutic strategies has become a critical area of research. In this context, the family of natural zeolites has emerged as a promising alternative to conventional therapeutics. Indeed, owing to their micro- and nano-structured porous nature, zeolites possess excellent ion-exchange and scavenging capabilities, considered to be crucial features in the design of anti-inflammatory and anti-microbial treatments. Additionally, zeolites could also have the potential to actively support the wound healing process by guiding cell behavior, proliferation, migration and differentiation. Moreover, zeolites can be easily extracted from volcanic and sedimentary rocks. Therefore, their intrinsic clinical features together with easy accessibility and abundancy make this inorganic material an optimal candidate as innovative therapeutic agent. Among the various types of zeolites, clinoptilolite was selected in this work for its therapeutic potential in treating chronic wounds. To further enhance its anti-inflammatory features particular attention has been focused on the functionalization of the powder through the implementation of a double step water-based reaction, finally leading to the surface exposure of heparin.

### Experimental

The clinoptilolite powder (referred to as MANC - Modified and Activated Natural Clinoptilolite) was kindly supplied by an external company (Froximun AG, Germany) and surface functionalized with heparin through a two-step reaction. First, MANC was subjected to silanization with 3-aminopropyltriethoxysilane (APTES) to expose amino groups. The reaction was optimized by studying the influence of solvent (i.e., water vs. toluene), reaction time (i.e., 3h vs. 24h) and drying process (i.e., room temperature vs. thermal treatment). Then, water-based carbodiimide chemistry was used to graft heparin through the formation of amide bonds between MANC-NH<sub>2</sub> and heparin-COOH. The success of functionalization was assessed through Dynamic Light Scattering (DLS), Infrared (IR) spectroscopy, Z-potential measurements and colorimetric assays to quantify grafted

molecules. In vitro cell viability and proliferation assays were conducted to study powder cytocompatibility in direct contact with murine fibroblasts, while the anti-inflammatory scavenging action exerted by heparin was quantified by measuring cytokines levels in the media over time. Lastly, a proof-of-concept of minimally invasive powder application was carried out by its embedding in a thermo-responsive hydrogel followed by temperature-controlled injection through needles with various dimensions in a wound-mimicking cavity.

## Results and Discussion

The as received MANC powder showed an average hydrodynamic diameter in the range 500-700 nm, a negative surface charge (i.e., Z-potential value approx. -50 mV) due to the exposure of -OH groups and a strong tendency to undergo sedimentation within few minutes after sonication. Powder silanization resulted in increased average hydrodynamic diameters and Z-potential values, confirming the success of APTES grafting, irrespective of adopted reaction parameters. The reaction time (i.e., 3h and 24 h) did not significantly alter the grafting yield. Conversely, solvent selection was found to strongly affect the number of exposed amino groups. More in detail, the superficial charge increased up to  $-21.35 \pm 1.44$  mV upon MANC functionalization in anhydrous toluene for 24h, but Z-potential trend inversion to positive values (i.e.,  $+19.68 \pm 0.80$  mV) was obtained only by using water as solvent. Such results were further confirmed by the colorimetric quantification of amino groups, found to be  $7 \times 10^{19} \pm 2 \times 10^{18}$  and  $1 \times 10^{20} \pm 3 \times 10^{19}$  units/g<sub>powder</sub> for reactions carried out in toluene and water, respectively. On the other hand, the use of organic solvent resulted in a more stable functionalization coating when the powder was put in contact with fluids, further enhanced by the thermal treatment as final drying step. Regarding water-based carbodiimide mediated heparin grafting, the success of the reaction was confirmed by: IR spectra through the appearance of the peaks at 1635 and 1530  $\text{cm}^{-1}$  ascribed to the C=O and N-H vibrations belonging to the amide bonds; Z-potential measurements giving negative values in accordance with the exposure of negative heparin molecules; increased particle average hydrodynamic diameter and, improved stability of the powder suspension over time. Moreover, the colorimetric quantification of heparin definitely confirmed the grafting, giving functionalization yields approx. equal to 100%. In addition, murine fibroblasts treated with functionalized MANC exhibited high cytocompatibility and improved cell proliferation compared to cells in contact with not-modified MANC. Moreover, preliminary tests showed heparin-grafted powder capability to sequester pro-inflammatory cytokines from the media, thus proving MANC potential as anti-inflammatory drug. Lastly, a proof-of-concept of the feasibility to encapsulate the powder in a thermo-sensitive hydrogel was carried out in its perspective clinical application as therapeutic agent in the form of injectable system or cream to be mini-invasively applied in the wound bed.

## Conclusions

Natural zeolites hold significant potential as alternative therapeutic agents for the treatment of chronic wounds owing to their inherent scavenging features. Furthermore, their wide availability, ease of extraction, and the feasibility of functionalization through environmentally friendly chemical processes further enhance their appeal as naturally derived therapeutic materials.

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