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JOURNAL OF RARE EARTHS, Vol. 29, No. 3, Mar. 2011, p. 259
JOURNAL OF RARE EARTHS
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Synthesis and characterization of lanthanum bonded agar-carbomer hydrogel: a promising tool for biomedical research

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Received 20 July 2010; revised 15 September 2010

Abstract: Agar-Carbomer (branched poly (acrylic acid)) hydrogel, an injectable bio-resorbable scaffold with a controlled nanostructure specifically designed for neural cell housing, was developed together with a new protocol for building three dimensional biohybrid cell/hydrogel systems. In order to overcome classic structural analysis inconveniences due to the high water amount, which affects instruments results and reliability, agar-Carbomer hydrogels were synthesized by microwave-assisted block copolymerization together with La^{3+} salts. Propylene glycol, glycerol and buffered saline solution were used as cross-linking agents and solvent, respectively. Biomaterial properties were not affected by the presence of lanthanum, and were checked via swelling and rheological analysis. Moreover, the presence of La^{3+} within the polymeric network was characterized by thermogravimetric analysis, environmental scanning electron microscopy and Fourier transformed infrared spectroscopy. The results showed that the rare earth presented uniform distribution in the hydrogel network due to the formation of chemical bonds after polymerization without being modified its luminescence emission spectrum that allowed hydrogel detection. These results made the obtained host-guest system a useful tool for analytical research studies concerning regenerative medical applications that could also be potentially taken up with *in vivo* experiments.

Keywords: hydrogel; acrylic acid; agarose; lanthanum; luminescence; drug delivery; rare earths

In recent years, growing interest has been given to regenerative medicine as it is considered to be the future path for the treatment of a very wide range of diseases and traumas[1,2]. Most promising approaches point towards the development of smart biological and pharmacological therapeutics which are able to provide *in situ* homing of drugs or cells, specifically aiming at efficient local delivery[3,4]. In this framework, injectable hydrogels capable of *in situ* polycondensation offer cell support, high drug release efficiency and very low systemic toxicity[5,6]. An *in situ* forming gel has an advantage over rigid scaffolds because it can conform to any shape and can be introduced using minimally invasive surgical procedures[7]. Literature suggests that these materials can be smart platforms that allow to overcome the classic disadvantages of local drug delivery and cell housing, thus opening new and very promising perspectives to advanced combined therapies based on topic delivery of cells and drug[8]. In this sparkling framework, this present work dealt with the development of a copolymeric injectable hydrogel that could easily convey and deliver both cells and drugs, being specifically thought for regenerative purposes in the field of spinal cord injuries[9–11]. Its synthesis involved two FDA approved polymers in a statistical block polycondensation: a synthetic branched polyacrylic acid (Carbomer 974P) and agarose, a common polysaccharide, both blended with appropriate crosslinkers in a water-based solvent[9].

As happens to all hydrogels, polymerization process is conducted in aqueous media. The presence of water, which gives the name to this class of polymeric systems, accounts for their high feasibility for biomedical applications. Otherwise, during experimental studies, relevant experimental problems may appear. Water entrapped into gel matrix often affects instrumental analysis, jamming the signal and thus compromising the study. Nevertheless, the necessity to surmount these limitations is mandatory. A precise knowledge and control over gel intimate structure is essential in order to design scaffolds to be able to achieve reliable performances into target tissues, i.e., desired gelation and degradation kinetics[8,12,13].

In this direction, the need of marking gel structure finds a possible solution in the employment of rare earths, which undeniably are used for this purpose in several polymeric formulations[14,15] due to their intrinsic characteristics, such as luminescence, selective absorption, optical transform, radiological shield, and electromagnetic response. Therefore, a hydrogel was here prepared by bonding with a rare earth. Lanthanum ion (La^{3+}) was used as a marker within the hydrogel, as it can be easily detected not only in *in vitro* analysis but also during *in vivo* experiments, particularly those where water signals from the gel system gets shaded by surrounding tissues, e.g., in MRI sessions[16]. Moreover, the use of La^{3+} plays another potentially fundamental role due to its high co-dopant ability[17]. It is indeed well known that highly efficient luminescent rare earths, like Eu^{3+} or Tb^{3+}, can show

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DOI: 10.1016/S1002-0721(10)60442-5

heavily constraining issues when barely included in polymeric networks[18,19]. Otherwise, the addition of La^{3+} allows a better dispersion of rare earths within the polymeric network, inhibiting cluster formation[19].

The aims of the present work are, thus, the structural investigation of this host-guest hydrogel-La^{3+} system and the demonstration of its suitability in analytical research studies concerning regenerative medical applications.

1 Experimental

1.1 Materials

Carbomer 974P was provided by Fagron (The Netherlands), triethylamine (TEA) with high purity was purchased from Fluka (Switzerland), and propylene glycol and glycerol were provided by Sigma-Aldrich (Germany). The solvent used was PBS (phosphate buffer saline), purchased from Sigma-Aldrich (Germany). Invitrogen provided Agarose, the other polymer involved in the reaction, while lanthanum nitrate hexahydrate was provided by Sigma-Aldrich (Germany). All materials were used as received.

1.2 Hydrogel synthesis and lanthanum loading

Hydrogel samples were prepared by bulk reaction in PBS at about 80 °C, where polymeric solution was achieved by mixing polymer powders into the selected solvent, adding a mixture of cross-linking agents made of propylene glycol and glycerol (along with tri-ethyl-ammine (TEA) for pH neutralization). Reaction pH was kept neutral. Polycondensation start was achieved by means of microwave (EM) stimulation. Key parameter in controlling in gelation reaction was the amount of hydroxyl groups available for reaction as they are the cross-linking sites, particularly those of propylene glycol (30% *w/w*), glycerol (0.5% *w/w*), to be reacted with those of Carbomer 974P (0.25% *w/w*) and agarose (0.25% *w/w*), altogether giving rise to the three dimensional matrix. Effective polycondensation was achieved by microwave heating (1 min per 10 ml of polymeric solution). The mixture was subsequently merged with a lanthanum nitrate-based solution (in deionized water 1 mg/ml) at a 50/50 volumetric ratio, placed in steel cylinders (0.5 ml each) and left to rest at 37 °C until reaching complete gelation at thermal equilibrium. The formation of esteric bonds between Agarose and Carbomer, which leads to the setting up of the hydrogel network, was described in previous works[14,15]. The stability of these materials is quite high and their degradation occurs via hydrolysis of ester bonds after several weeks. Since such characteristic time is much longer than the experimental time, degradation reactions are thus fully neglected in this work. Agar-Carbomer gels were previously called with the *AC* acronym[9–11], and in the presence of lanthanum salt, here became *LAC1*.

1.3 Physical characterization

1.3.1 Swelling characterization The hydrogel samples were synthesized, then freeze-dried for 24 h, weighed (W_d) and poured in excess of PBS to achieve complete swelling at 37 °C in 5% CO_2 atmosphere; such conditions were considered because typical of *in vitro* cellular biology experiments. The swelling kinetics was measured gravimetrically. The samples were removed from PBS at regular times. Hydrogel surfaces were then wiped with moistened filter paper in order to remove the excess of solvent and then weighed (W_t). Swelling ratio is defined as follows:

$$\text{swelling ratio} = \frac{W_t - W_d}{W_d} \cdot 100 \quad (1)$$

where W_t is the weight of the wet hydrogel as a function of time, and W_d is the weight of the dry hydrogel as evaluated after freeze-drying. The swelling equilibrium is the maximum value reached by its kinetic[9].

1.3.2 Rheological measurements Rheological analysis on gel samples were performed at 37 °C using a Rheometric Scientific ARES (TA Instruments, New Castle, DE, USA) equipped with parallel plates of 30 mm of diameter and a 4 mm gap between them.

1.3.3 Thermogravimetric analysis Thermogravimetric analysis (TGA) measurements were conducted with a Netzsch TG 209 instrument in air atmosphere. Thermograms were taken in the range of 30–1 000 °C. The mass loss of the samples as a function of temperature was followed at a heating rate of 10 °C/min.

1.3.4 Morphological studies: environmental scanning electron microscopy analysis ESEM analysis was performed with Evo 50 EP Instrumentation (Zeiss, Germany). In order to preserve the actual morphology of the hydrogel under complete swelling, freeze-drying (24 h) was applied to remove all the liquid phase by sublimation. Due to the low operating values of temperature and pressure, the polymer chains were expected to retain the same conformation they had in wet conditions[9].

1.3.5 Fourier transform-infrared (FT-IR) spectra Hydrogel samples, after being left to soak for 24 h in excess of solvent, were freeze-dried and laminated with potassium bromide. FT-IR spectra were recorded to assess the presence of lanthanum within the polymeric network using a Thermo Nexus 6700 spectrometer coupled to a Thermo Nicolet Continuum microscope equipped with a ×15 Reflachromat Cassegrain objective.

1.3.6 Luminescence capability Hydrogel-La^{3+} system emission and absorption spectra were recorded with a Cary50 Varian spectrophotometer. The analysis was conducted between 400 and 1 000 nm.

2 Results and discussion

During swelling of a three-dimensional hydrogel network, polymer chains assume a stretched conformation but, by increasing the elongation, an elastic force acts in the opposite direction; this force limits the stretching process. On the other hand, polymer swelling is promoted by the poly-

mer-solvent mixing process, which lowers the total free energy by increasing system entropy. The action of these opposite forces leads to the attainment of a thermodynamic equilibrium, i.e., swelling equilibrium. Experimental data showed (Fig. 1) that swelling equilibrium is reached within about 4 h, i.e., our *LAC1* gel belongs to the group of the so-called "superabsorbent" hydrogels. This swelling behavior and its maximum value are in complete accordance with bare *AC* hydrogels[9] and they represent the first essential properties for being good scaffolds for tissue engineering[20,21].

As explained above, thixotropy is the main requisite for an *in situ* forming hydrogel, as it is important for gel injectability, which is the second essential property of hydrogels to be used as low invasive scaffolds. Pseudoplastic nature of Agar-Carbomer hydrogels was already described in a previous work[9,10], demonstrating the influence of cross-linking agents on physical properties. In this work, nevertheless, the interest was also focused on confirming the thixotropic behavior also in the presence of lanthanum. Furthermore, thixotropy also plays a fundamental role in a drug-delivery contest allowing to achieve high clinical efficacy of the pharmaceutical formulation[22], by contributing their extended retention time at the target site and enhancing systemic drug bioavailability.

Fig. 2 shows shear behavior plots of *LAC1* gel, which present the typical hysteresis loop, distinguished marks of thixotropic materials. Investigated formulation showed thixotropy at low shear rate values. The hysteresis loop represents the energy loss required in obtaining the sol-gel transition and is directly linked to the time necessary for material rearrangement[23]. Lanthanum presence could act as an accelerator of sol-gel transition, as it interposes between polymer chains and slows down network rearrangement. Most probably, this effect is deadened by lanthanum ability to coordinate saccharide units[24]. The intrinsic discontinuity of the network due to the presence of La^{3+} atoms is well balanced by its ability to be assembled within the structure.

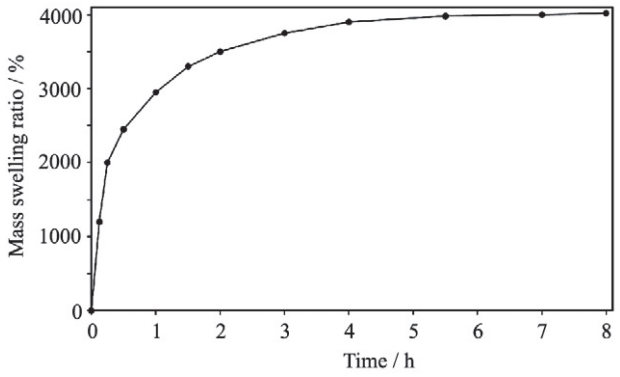

Fig. 1 Maximum swelling and swelling kinetics of the lanthanum bonded hydrogel in PBS at 37 °C

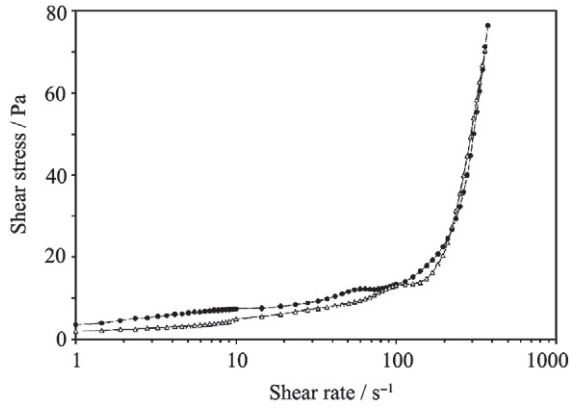

Fig. 2 Thixotropic loop of *LAC1* gel sample

As seen, the presence of lanthanum does not influence the hydrogel as biomedical material, but in order to complete the present investigation, it is necessary to precisely assess its presence inside the hydrogel network. Its role in thermal degradation was firstly investigated and the related thermograph is presented in Fig. 3. Concerning the lanthanum nitrate decomposition, it is possible to single out two different steps[25]: the first takes place under 325 °C, associated with water emission, while the second one occurs above 325 °C, with NO_2 and O_2 emissions. These two different stages correspond to dehydratation and further decomposition. The other steps present in the plot are due to the presence of polymeric C–C network and are related to its oxidative degradation. In accordance with theoretical values[24], the weight loss observed at 1 000 °C is around 10%. For the sake of completeness, it has to be said that the presence of lanthanum did not significatively affect the hydrogel behaviour around the typical *in vivo* temperature, i.e., about 37 °C.

In order to preserve morphology of the hydrogel after complete swelling, freeze-drying was applied to remove all the liquid phase by sublimation. Due to the low operating values of temperature and pressure, the polymer chains are expected to retain the same conformation they had in wet conditions[26]. The presence of lanthanum was then confirmed by elemental analysis performed during ESEM scans, as it can be observed from Fig. 4 where some of lanthanum ions are pointed out by green arrows in Fig. 4 (b) and (d).

As shown by the general overview of Fig. 4 (a), *LAC1* gel appears to have a very close and compact matrix, with high degree of regularity and well-defined network. Focusing on the presence of lanthanum, it can be noted that it is very well detectable from detailed views (Fig. 4 (b), (d)) and elemental analysis (Fig. 4 (c)). These results confirm the presence of bonds between the rare earth and the polymeric network.

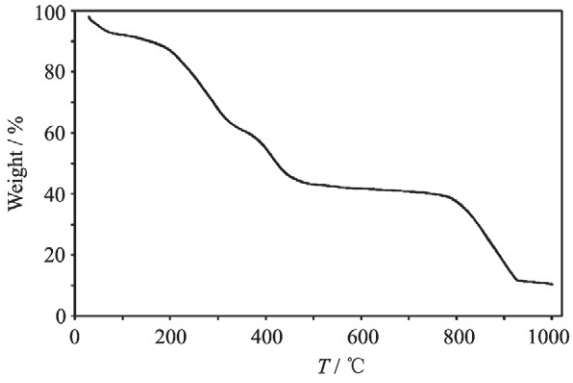

Fig. 3 Thermal degradation of *LAC1* gel sample

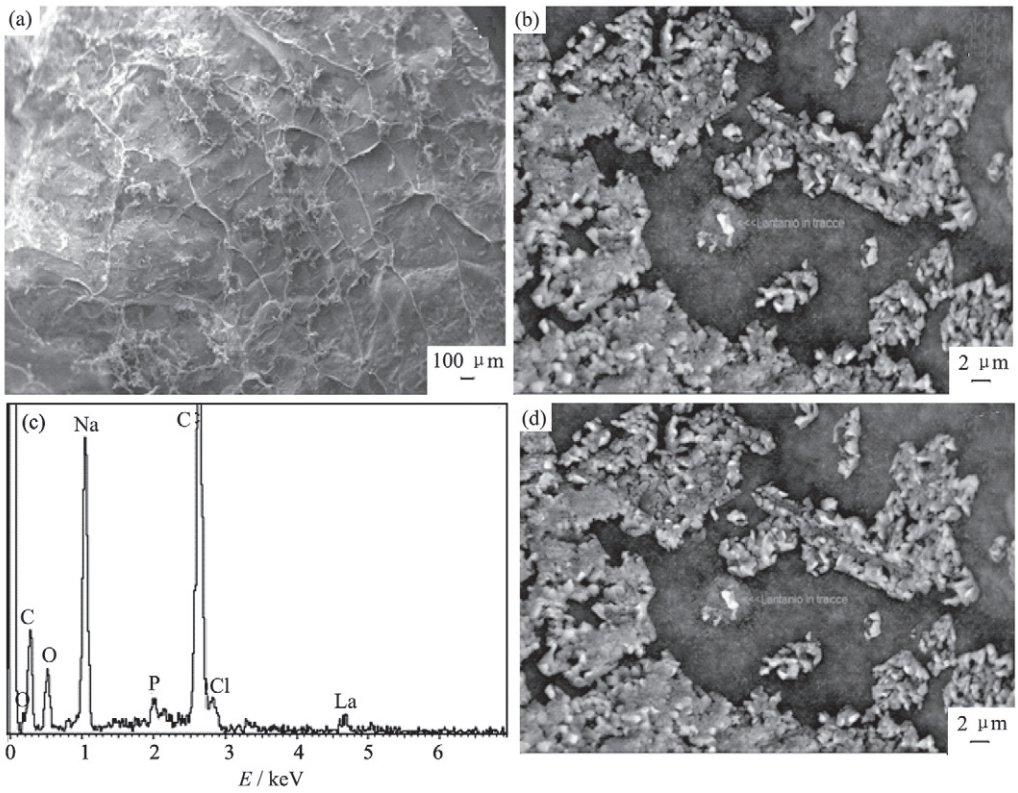

Fig. 4 ESEM images of *LAC1* at different magnifications: 29× (a), 2000× (b, d), and the elemental analysis of *LAC1* (c)

This aspect is extremely relevant and deserves to be pointed out, particularly with respect to other La^{3+} or, in general solute loaded systems described in literatures[17,27,28]. Loaded hydrogels usually have atoms dissolved into water, and thus free to diffuse outside the gel matrix, and not linked to gel polymeric network in a stable, or hemistable manner[27,29]. This does not allow to use lanthanum ions to investigate hydrogel structure. In the case of *LAC1* gel, on the other hand, lanthanum is directly bonded to the polymeric matrix of the hydrogel and thus its presence can be used to investigate gel network. Here, following elemental analysis, the formation of lanthanum cross-links within the hydrogel was assessed also via FT-IR spectroscopy. Fig. 5 shows the FT-IR spectra of *LAC1* gel. The spectrum shows a broad peak of around 3 450 cm^{-1}, which is due to the stretching vibration of O–H bonds, while peaks around 2 940 cm^{-1} are due to the C–H stretch. The formation of esteric bonds is visible by peaks corresponding to symmetric (around 1600 cm^{-1}) and asymmetric (around 1 400 cm^{-1}) CO_2 stretches. Moreover, peaks around 1 080 cm^{-1} are related to C–N vibration, confirming the presence of TEA inside the network. Spectra also show peaks related to C–O–C stretch vibration, in the range of 800–820 cm^{-1}, which represents the glycosidic bond between monosaccharides (typical of agarose structure).

The building blocks, or subunits, of macromolecules form a stable structure made up mostly of C–C bonds, usually referred as the "carbon skeleton". C–C and C–H bonds are said to be non polar and thus tend to be lowly reactive. Building blocks of macromolecules act as discrete subunits because their internal structure consists of C–C bonds; C–O and/or C–N bonds make the links between the subunits. Indeed, degradable bonds generally involve oxygen or nitrogen atoms. Here, the obtained FT-IR results allow stating that the most important groups in the studied gel are: –OH, C–H, CO_2, C–N, vinyl, and C–O–C. Moreover, the spectrum reveals also a number of peaks in the NO_2 stretching region (bidentate peak at 2 904 cm^{-1}, single peak at 1 643 cm^{-1}, unidentate peak at 1 276 cm^{-1}) and in the NO stretching region (1 042 cm^{-1}) suggesting the presence of nitrate ions[30]. These last findings from IR spectra prove and confirm the formation of lanthanum cross-links with the native gel structure.

Literatures describe other La^{3+}-hydrogel systems[17,31] and very often the solute loaded does not present its characteristic luminescence, i.e., that as La^{3+} ion, and this is mainly due, as described before, by its being freely dissolved into the water and thus having a signal shaded by the gel polymeric

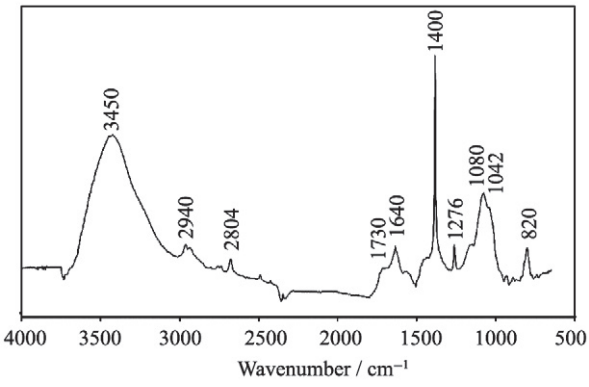

Fig. 5 Fourier transform infrared spectrum of *LAC1*

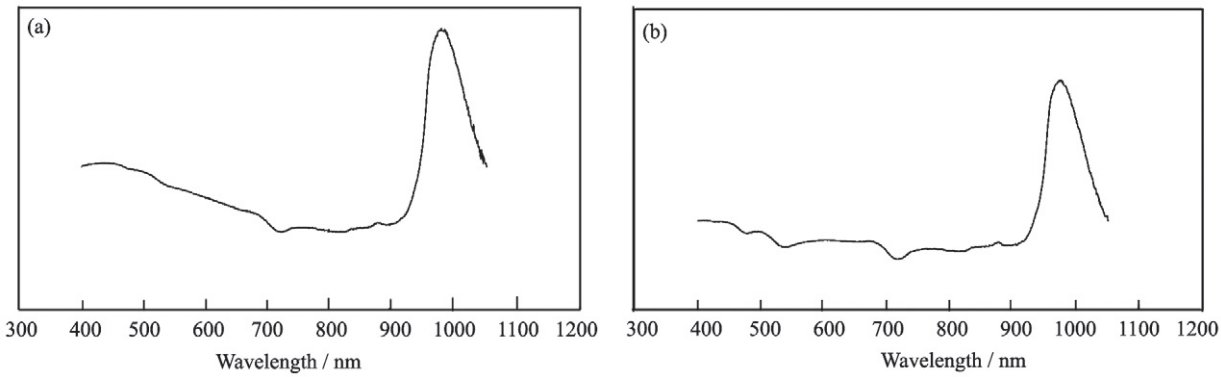

Fig. 6 Emission spectra of *LAC1* (a) and lanthanum nitrate (b)

backbone[17,31]. Here, on the other hand, the emission spectrum of *LAC1* exhibits a strong luminescence at 970 nm, exactly the same visible in the lanthanide salt spectrum. This is the second very relevant aspect that deserves being noted and can be explained because the gel polymeric network does neither overcome nor cover the optical signals that come from lanthanum as they are cross-linked together.

3 Conclusions

Investigating hydrogel structure is essential when designing smart tools for regenerative medicine, but, as stated in their name, hydrogels are characterized by a very high presence of water that inhibits most of traditional methods used to investigate their structures. Here, an indirect method was applied to overcome these disadvantages. A promising biomedical gel was marked with a lanthanum salt obtaining a direct crosslink between La^{3+} and polymeric backbone. The presence of the rare earth was confirmed and characterized by means of different analytical techniques. TGA showed lanthanum presence and influence, ESEM showed that the rare earth was homogeneously bonded, while FT-IR confirmed the La^{3+}-gel bonding and revealed its chemical nature. Moreover, beside its presence, $La(NO_3)_3$ employment within hydrogel formulation did not affect essential scaffolding features, such as swelling ability and thixotropy. La^{3+} ions were indeed homogeneously dispersed within the matrix, and form stabile bonds with polymeric chains, giving rise to a uniform structure, without local discontinuities. Moreover, rare earth presence and its unmodified luminescence allowed to better and more clearly identified hydrogel matrix and this can be of great advantage not only in *in vitro* investigations, but also in an *in vivo* contest, where water could be very problematic for scaffold detection.

The use of lanthanide salts in hydrogel studies, and more generally during the development of water based biocompatible scaffolds, is therefore promising. Here, in the case of *AC* gels, lanthanum bonding not only did not affect essential matrix properties but also enhanced its experimental detection. Luminescence properties indeed, very useful and always more commonly applied in biomedical research, did not change due to the presence of the hydrogel. This property, together with its good biomedical performances, made the obtained host-guest hydrogel-La^{3+} system *LAC1* a useful tool for analytical research studies concerning regenerative medicine applications that could potentially also be taken up to *in vivo* experimentations.

Acknowledgments: The authors would like to thank Davide Gulotta (Department of Chemistry, Materials and Chemical Engineering "Giulio Natta"), Nicola Martino and Maria Claudia Sanarelli (Department of Physics) of the Politecnico di Milano for their help in FT-IR and optical analyses.

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