FRET: A tool to study the interaction between apatite and collagen?

Isabelle Dos Santos [a], Serge Mazères [b], Michèle Freche [c], Jean-Louis Lacout [c], Anne-Marie Sautereau [a,*]

[a] PPB, Institut Carnot CIRIMAT UMR 5085, Service de Pharmacie galénique, Faculté de Pharmacie, Université Paul Sabatier, 118 route de Narbonne, 31062 Toulouse cedex 4, France
[b] Institut de Pharmacologie et de Biologie Structurale, IPBS—CNRS, 205 route de Narbonne, 31077 Toulouse cedex 4, France
[c] PPB, Institut Carnot CIRIMAT UMR 5085, ENSIACET/INPT, 118 route de Narbonne, 31077 Toulouse cedex, France

A B S T R A C T

Fluorescence Resonance Energy Transfer, FRET, is largely used in biology to demonstrate the occurrence of molecular interactions. In order to investigate the vicinity between collagen and calcium phosphate in bone substitute biomaterial, composites constituted of autofluorescent collagen and apatite doped by europium were synthesized by a precipitation method. The first results show a fluorescence energy transfer between the two fluorescent components and are very promising.

Keywords:
FRET
Luminescence
Composite materials
Apatite
Collagen
Molecular interaction

1. Introduction

The composition of human bone is a well-known mineral/organic hybrid consisting of hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) and organic (mainly collagen) constituents. Apatite/collagen composites are probably the most biomimetic system for osseous replacement or regenerative therapy [1–9]. These materials based on inorganic and natural macromolecules are not easily characterized, and particularly the chemical interaction between apatite and collagen has been poorly studied. In general, Fourier Transform Infrared (FTIR) analysis was carried out and the authors [4–9] have shown that the COO^- band of collagen was shifted due to the interaction with apatite. Nevertheless, this band is very weak and the low value of the shift from 1340 to 1337 cm^{-1} is not very significant. Modified thermogravimetric analysis (TG-DTG), Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM) observations were also reported [4–9].

A new approach of the study of collagen/apatite interaction is proposed: the Fluorescence Resonance Energy Transfer (FRET). FRET (sometimes called Förster Resonance Energy Transfer), is frequently used in biology to characterize the interactions of biomolecules [10,11]. The detection of a significant FRET signal is a strong indicator of the nanometer range proximity of two fluorophores. In our case, the first component, collagen is autofluorescent. The native fluorophore is thought to be pyridinoline, one of the major cross-links within the primary structure of collagen fibrils [12]. Calcium phosphate apatite is not naturally fluorescent, so, it is doped with the rare earth, europium, Eu^{3+}. Many studies have shown that Eu^{3+} can partially substitute for Ca^{2+} ions in natural or synthetic apatites [13].

2. Experimental

Europium doped apatite with Eu/(Eu+Ca)=2% (atoms) is synthesized by a precipitation method in an aqueous medium at 37 °C [adapted from 13]. Calcium nitrate [$Ca(NO_3)_2{\cdot}4H_2O$] (Merck), 19,5 mmol, and europium nitrate [$Eu(NO_3)_3{\cdot}6H_2O$] (Alfa Aesar), 0,4 mmol, are dissolved in 80 ml of deionized water (solution A). Diammonium hydrogen phosphate [$(NH_4)_2HPO_4$] (Carlo Erba), 11.9 mmol, are dissolved in 200 ml of deionized water. The pH solution is adjusted in the range of 8–9 with ammonia (solution B). Solution A is quickly added to solution B at 37 °C while stirring. After 2 h, in the same conditions of pH, temperature and stirring, the obtained precipitate is filtered, washed with deionized water and freeze-dried for 48 h.

Two types of compounds are prepared. The first one, reported as "mixture" is prepared by mixing the europium-doped apatite with collagen (type I from bovine Achilles tendon, Sigma Aldrich) using an apatite/collagen weight ratio fixed at 90/10. The second, a Eu-doped apatite/collagen, reported as "composite", is synthesized in the same way of the apatite previously described, except that collagen is firstly dispersed in the solution B. In fact, in this case, according to these synthesis conditions, europium-doped apatite is precipitated in a suspension of collagen.

All the samples are prepared in pellets form by compression in a mold.

* Corresponding author. Tel./fax: +33 562256841.
E-mail addresses: asautere@cict.fr (I. Dos Santos), serge.mazeres@ipbs.fr (S. Mazères), Michele.Freche@ensiacet.fr (M. Freche), JeanLouis.Lacout@ensiacet.fr (J.-L. Lacout).

doi:10.1016/j.matlet.2008.07.035

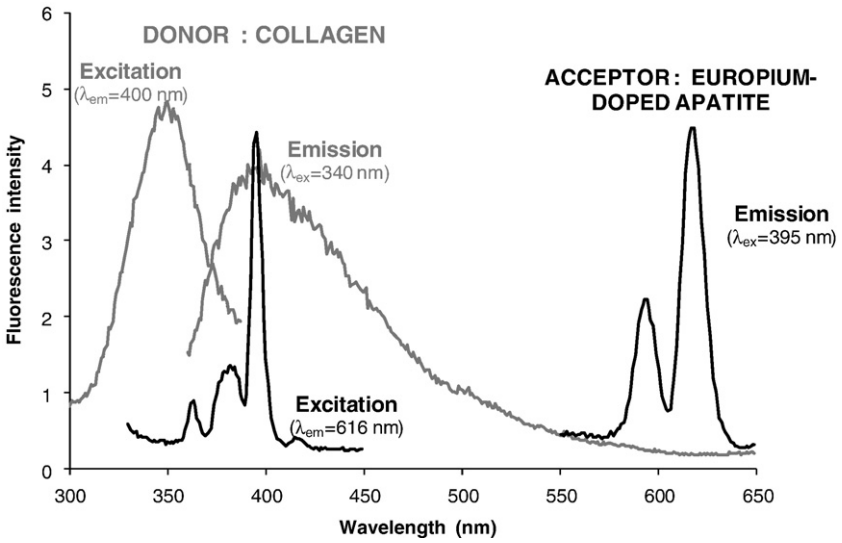

Fig. 1. Fluorescence excitation and emission spectral profiles of the donor, collagen, and the acceptor, europium-doped apatite.

Due to the synthesis route of the composites, in order to control and compare the affinity of europium for apatite or collagen, two reference samples are prepared. Europium nitrate in the ratio (Eu/(Eu+Ca): 2% (atoms) is added on one hand to a suspension of pure apatite and on a second hand to a suspension of collagen. The suspensions are maintained for 2 h, at pH 8–9 and 37 °C. After filtration, the solid samples are washed with deionized water and freeze-dried.

Fluorescence excitation and emission spectra are performed on an Aminco SPF 500 C spectrofluorimeter. The spectra are recorded using a special sample holder design for solid state substrates fixed on a microscope glass plate.

3. Results and discussion

Aside from spatial proximity, it is well-known that for efficient FRET to take place the fluorophores must exhibit a significant overlap of the donor's emission spectrum with the acceptor's excitation spectrum. The emission band of collagen, which is the donor, overlaps with the excitation band of europium-doped apatite, the acceptor (Fig. 1).

Moreover, after excitation at 340 nm, no signal is detected for collagen between 550 and 650 nm, the emission area of europium, and europium itself is not subject to direct excitation at the collagen excitation wavelength. In these conditions, if collagen and doped apatite are sufficiently close to each other, FRET will occur. After fluorescence excitation of collagen at 340 nm, the fluorescence emission of collagen would decrease and in the same time europium-doped apatite would emit, two characteristic peaks would be detected at 593 nm and 616 nm.

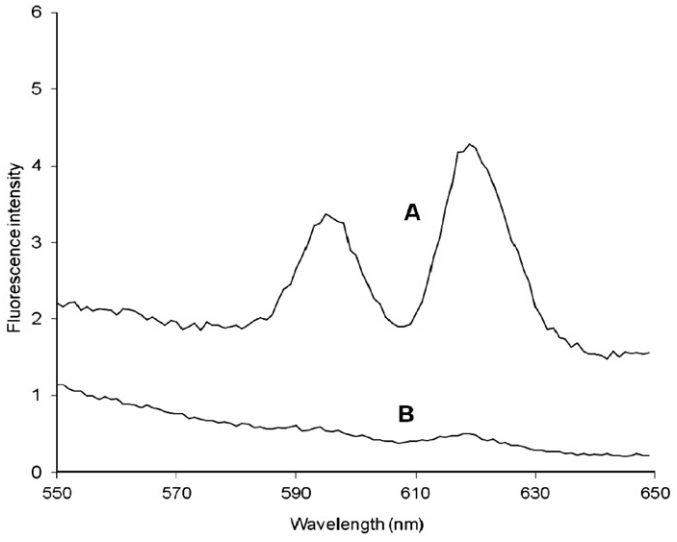

Fig. 2. Fluorescence emission spectra of europium mixed with apatite (A) and mixed with collagen (B), λ_{ex}=395 nm.

Many studies on europium-doped apatite were reported but no synthesis was carried out with collagen. The europium affinity for apatite and collagen is tested by mixing europium on one hand to apatite and on other hand to collagen. The fluorescence emission spectra of the samples after excitation at 395 nm (the maximum excitation wavelength of europium) are reported on Fig. 2. The intensity of the signal in the case of apatite is significantly higher than in the case of collagen. Europium has a more significant affinity for apatite than for collagen.

The comparison between the emission spectra of europium-doped apatite/collagen mixture and the corresponding composite after excitation at 340 nm shows emission peaks of europium at 593 nm and 616 nm only for the composite (Fig. 3). This result reveals fluorescence energy transfer from collagen to europium. It is fully in agreement with the composite synthesis methodology which could allow closer interaction between collagen and apatite.

Different amounts of europium in the composite are tested such as the Eu/(Eu+Ca) ratio varies in the range of 0–5%. Fig. 4 exhibits the fluorescence emission spectra at 340 nm of the various composites. The fluorescence energy transfer is observed with the two emission peaks at 593 nm and 616 nm. The intensity of the peaks lightly increases with the europium content till the Eu/(Eu+Ca) ratio equals 2. Above this value the intensity does not apparently increase. One explanation may be the quenching process due to high concentrations of europium. These results show that the ratio of europium in apatite must not exceed 2–3%. Moreover, this limit is in agreement with previous works concerning the introduction of europium by precipitation in the apatitic lattice [13]. Above this value the apatitic compounds are not homogeneous and europium is in an extra phase.

It is worth noting that the emission spectra of the donor, collagen, are modified (Figs. 3 and 4). A decrease of fluorescence intensity is observed at 395 nm, the wavelength value corresponding to the maximum of europium excitation. This partial quenching of emission spectra of the donor increases with the europium ratio. A decrease of fluorescence intensity in the area of spectra overlap is typically due to fluorescence energy transfer by radiative process i.e. via emission of a photon from the donor and its re-absorption by the acceptor. By FRET, a non-radiative fluorescence energy transfer, the emission spectra of the donor decrease at the same rate whatever the emission wavelengths. A radiative transfer can occur even if the two fluorophores are not in nanometer range proximity. However, radiative and non-radiative transfer could occur concomitantly which could explain the fluorescence energy transfer only observed in the case of composites. Nevertheless, no emission peaks from the europium-doped apatite/collagen mixture could also be due to an environment and a physic state different from the composite.

4. Conclusions

FRET is potentially very interesting to obtain information on the molecular interaction between collagen and apatite. Such results may be preciously helpful for the development of bone-like composites. Collagen and europium-doped apatite have all the spectral properties to be respectively donor and acceptor of fluorescence in the FRET process. These first results on the composites are very promising, they show fluorescence energy transfer but undeniably radiative transfer is

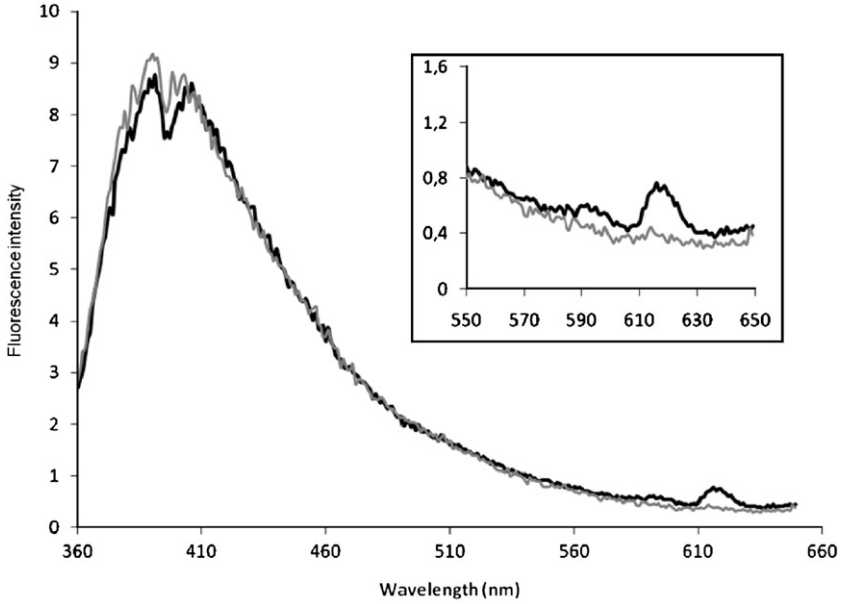

Fig. 3. Fluorescence emission spectra of europium-doped apatite/collagen mixture (—) and composite (▬) λ_{ex} = 340 nm.

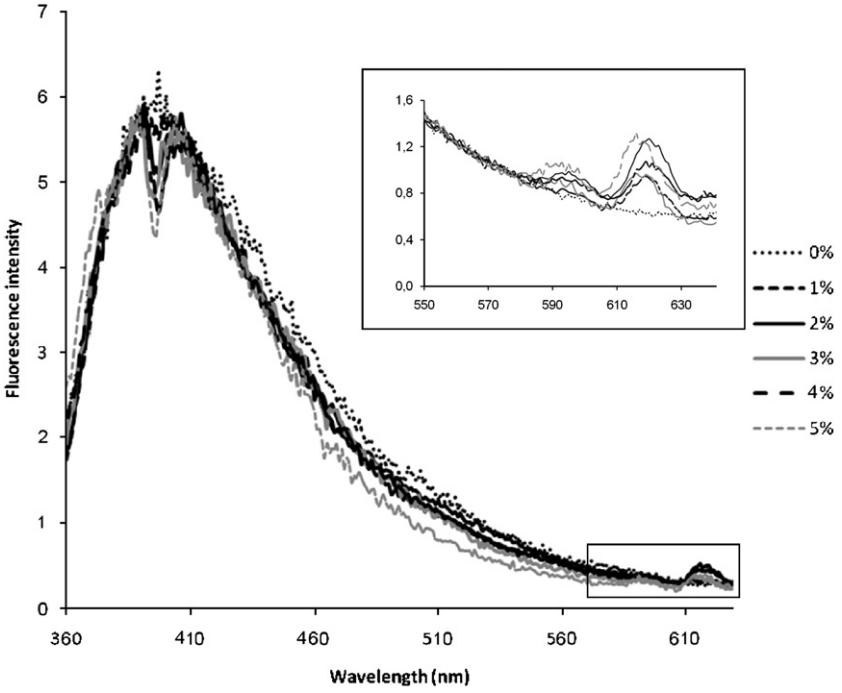

Fig. 4. Fluorescence emission spectra of composites Eu/(Eu+Ca): 0–5%, λ_{ex} = 340 nm.

detected. Further investigations specific to FRET are required. Studies using the techniques of time resolved fluorescence and fluorescence lifetime imaging microscopy are actually in progress.

References

[1] Rhee SH, Lee JD. J Am Ceram Soc 2000;83:2890–2.
[2] Kikuchi M, Itoh S, Ichinose S, Shinomiya K, Tanaka J. Biomaterials 2001;22:1705–11.
[3] Zhang W, Liao SS, Cui FZ. Chem Mater 2003;15:3221–6.
[4] Roveri N, Falini G, Sidoti MC, Tampieri A, Landi E, Sandri M, Parma B. Mater Sci Eng C 2003;23:441–6.
[5] Tampieri A, Celotti G, Landi E, Sandri M, Roveri N, Falini G. J Biomed Mater Res 2003;67:618–25.
[6] Kikuchi M, Ikoma T, Itoh S, Matsumoto HN, Koyama Y, Takakuda K, et al. Comp Sci Technol 2004;64:819–25.
[7] Lin X, Li X, Fan H, Wen X, Lu J, Zhang X. Mater Lett 2004;58:3569–72.
[8] Murugan R, Ramakrishna S. Comp Sci Technol 2005;65:2385–406.
[9] Wang Y, Yang C, Chen X, Zhao N. Adv Eng Mater 2006;8:97–100.
[10] Bazin H, Preaudat M, Trinquet E, Mathis G. Spectrochim Acta Part A 2001;57:2197–211.
[11] Hallworth R, Currall B, Nichols MG, Wu X, Zuo J. Brain Res 2006;1091:122–31.
[12] Fujimoto D, Moriguchi T. J Biochem 1978;83:863–7.
[13] Doat A, Pelle F, Gardant N, Lebugle A. J Solid State Chem 2004;177:1179–87.