EUROPEAN SYNCHROTRON RADIATION FACILITY

INSTALLATION EUROPEENNE DE RAYONNEMENT SYNCHROTRON

ESRF

Experiment Report Form

The double page inside this form is to be filled in by all users or groups of users who have had access to beam time for measurements at the ESRF.

Once completed, the report should be submitted electronically to the User Office using the **Electronic Report Submission Application:**

http://193.49.43.2:8080/smis/servlet/UserUtils?start

Reports supporting requests for additional beam time

Reports can now be submitted independently of new proposals – it is necessary simply to indicate the number of the report(s) supporting a new proposal on the proposal form.

The Review Committees reserve the right to reject new proposals from groups who have not reported on the use of beam time allocated previously.

Reports on experiments relating to long term projects

Proposers awarded beam time for a long term project are required to submit an interim report at the end of each year, irrespective of the number of shifts of beam time they have used.

Published papers

All users must give proper credit to ESRF staff members and proper mention to ESRF facilities which were essential for the results described in any ensuing publication. Further, they are obliged to send to the Joint ESRF/ ILL library the complete reference and the abstract of all papers appearing in print, and resulting from the use of the ESRF.

Should you wish to make more general comments on the experiment, please note them on the User Evaluation Form, and send both the Report and the Evaluation Form to the User Office.

Deadlines for submission of Experimental Reports

- 1st March for experiments carried out up until June of the previous year;
- 1st September for experiments carried out up until January of the same year.

Instructions for preparing your Report

- fill in a separate form for each project or series of measurements.
- type your report, in English.
- include the reference number of the proposal to which the report refers.
- make sure that the text, tables and figures fit into the space available.
- if your work is published or is in press, you may prefer to paste in the abstract, and add full reference details. If the abstract is in a language other than English, please include an English translation.

ESRF	**Experiment title:** **Synchrotron X-ray micro fluorescence characterization and labeling of cellulosic ether used for injectable bone substitutes**	**Experiment number**: CH-1464
Beamline: 18F	**Date of experiment**: from: 09-APR-03 to: 12-APR-03	**Date of report**: 21-july-03
Shifts: 9	**Local contact(s)**: Sylvain BOHIC	*Received at ESRF:*

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Report: This is a preliminary report because all the data was no traited yet.

The aim of this work was to study the labeling of cellulosic ethers for biologic experiments, using Synchrotron X-ray micro-Fluorescence. Current approaches to the reconstruction of bone tissue in orthopedic surgery, stomatology and dental applications rely on calcium-phosphate (CaP) ceramics. CaP biomaterials are used in bone repair, substitution or augmentation, as osteoconductive fillers to achieve bone coalescence. CaP are the principal raw materials used for bone substitutes in the elaboration of granules or blocks. The macroporosity of CaP blocks facilitates the penetration of cells and biological fluids into the implant, allowing the osteogenic process to occur within the inner surface of pores. Injectable CaP injectable biomaterials should associate efficient bone colonization and implantation with non-invasive surgical techniques. The Injectable bone substitute (IBS) consists of CaP ceramic granules suspensions in water soluble polymer carrier phase and is developed in our laboratory (patent WO-9521634, 17/08/95). The bone ingrowth in the suspension is very fast and CaP granules in the polymer carrier phase create a so-called “interconnected macroporosity”.Our choice was made on the development of an injectable ceramics and more particularly of a material ready to use without any handling nor mixture on behalf of the user, the surgeon. We chose synthetic polymers derived from cellulose apparently of the candidates of choice for the realization of a material composite, injectable, biocompatible and ready to use. The three-dimensional microtomographic technique, made at ESRF in 2001 (1 publications and 1 accepted), which was used for the first time in this study to describe bone colonization of CaP biomaterials, confirmed the intimate bone bonding ability of the biomaterial resulting from their bioactive properties. It also showed that bone ingrowth developed extensively in the intergranular spaces of ceramic, suggesting that the final mechanical properties of the composite tissue are similar to those of initial host bone structure.

The question that was not answered yet is : What about the polymer after implantations? Is it still in implant, in bone matrix, is it metabolized, degredated by cells or simply diluted and carried in blood circulation before elimination.

There is no literature about implantation of cellulosic ethers other than ophthalmic medical devices and because they remove it during surgery nobody was interested in this question. Radioactive labeling is difficult, with specific synthesis’s. After radioactive labeling, manipulations of the biomaterial and in vitro and in vivo experiments need specific installations and procedures no described (literature) in our conditions for biomaterials or medical devices.

The aim of this first ESRF experimentation was to control the detection of Ru by micro-fluorescence and the labeling of HPMC.

Experimental method

In the context mentioned above, our purpose is to label the cellulose ether with a derivative of tris-(2,2'-bipyridine)ruthenium(II) complex [Ru(bpy)2(bpyR)2+] covalently attached to the cellulosic network. As ruthenium is not present in biological fluids, it could be used as a probe to be detected by X-ray micro-fluorescence at ESRF (Ru Kα line : 101.07 KeV?) on microtomographic bone cuts after implantation, in order to determine the degradation pathway of the cellulosic phase. The stability of the polypyridil ruthenium complex and of its chemical linkage to the cellulose ether should avoid any non-specific dissemination.

Experimental 1: $[Ru(bpy)_3]Cl_2.xH_2O$ complex in water solution with different Ru ratio from 0.1 ppm to 200ppm. These solutions were analysed in special glass capillaries.

Experimental 2: $[Ru(bpy)_3]Cl_2.xH_2O$ complex in polymer (HPMC E_4M à 3% (w/w) water solution with different Ru ratio from 1ppm to 200ppm. These solutions were analysed after drying on a plastic film.
A part of these solutions were dialysed in dialys tubes to know if Ru complex could be associated to the polymer withoutcovalent linkage: The polymer solutions were analysed after drying on a plastic film and the dialysats were analysed in capillaries.

Experimental 3: The next polymer solutions with different amounts of $[Ru(bpy)_3]Cl_2.xH_2O$ were blend with BCP granules (50% w/w) to make IBS and to know if Ru complex is always detected. A part of these blends were dialysed in dialys tubes to know if Ru complex is adsorbed on the BCP surface : The suspensions were analysed after drying on a plastic film and the dialysats were analysed in capillaries.
All samples were tested before and after steam sterilisation.

Experimental 4: Labeling polymer

For this experimentation we used the polymer HPMC silanised (Si-HPMC) :E4M (Colorcon-Kent-England) with 14,24 % of 3-glycidoxypropyltrimethoxysilane (GPTMS) graft (0.78 % of Silicium / Dry polymer).
In basic medium, the grafts silanes are ionized and the chains are disjoined, then when the pH decreases the number of protons increases and R-Si-O becomes R-Si-OH very reactive. Condensation of R-Si-OH groups with acidic condition will form a three-dimensional network and create a hydrogel. For labeling the polymer, we used this property and we synthetized a $[Ru(bpy)_2(bpy\text{-}R\text{-}Si\text{-}(O\ Et)_3]$ (Si-Ru).

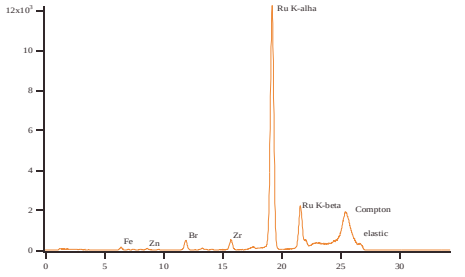
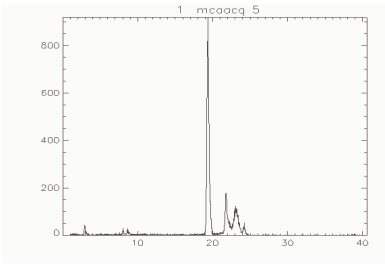
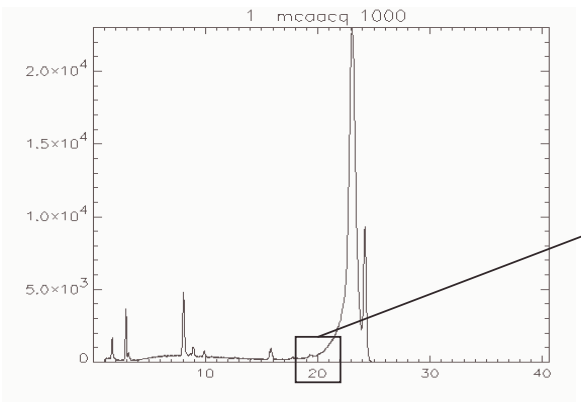
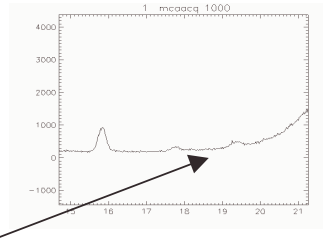


Figure: 1



1e_002 : Aqueous solution of Ru(bpy)3Cl2 in 200 ppm ratio of Ru (capillary / 1000s of aquisition)





1e_002 : Aqueous solution of Ru(bpy)3Cl2 in 0,1ppm ratio of Ru (capillary / 1000s of

A blend of Si-HPMC and Si-Ru was performed with to different concentrations of Ru (0.1 eq/Si and 0.01 eq/Si) in sodium hydroxyde with a pH of 13. An Heppes buffer were added to decrease the pH and the self cross-link can occurs. We obtain different labelled gels. Some of the labelledpolymer were blend with BCP granules before the reaction to obtain a composite of the labelled polymer.
Each sample were put in dialyses bags and the dialysats were analysed.

The samples were analysed after be freezed? and cut with cryotome to approach the detection limits of the method and control the uniformity of the blend.

Results:

More than 60 samples were analysed and for each sample a minimum of 10 mesurements was made. All the results for the quantification are not performed yet and are in process.

In a first step we determined the sensitivity limit of the micro-fluorescence detection and we confirmed that it was about 0.1 ppm (Figure 1)

When a very low amount of the ruthenium-based label is blend with the cellulosic ether, the detection is no affected but the results is non uniform on the film.

The Ru complex doesn't stay associated to the polymer without covalent linkage because it diffuses across the dialyse? membrane.

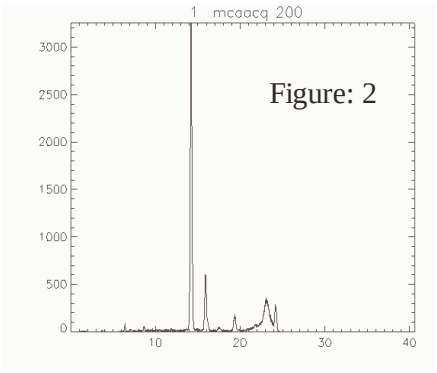
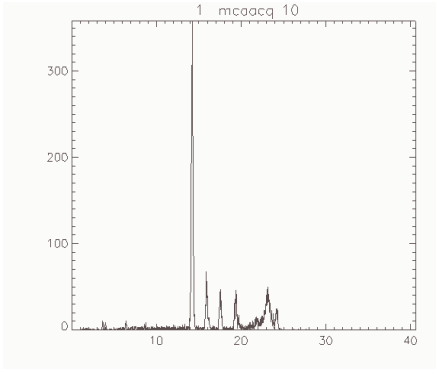


Figure: 2

3c1_002 : Polymer solution + BCP with 5ppm of Ru (cryostat)



3d1f_010: Polymer solution + BCP 0,5ppm of Ru (film)

When a very low amount of the ruthenium-based label is blend with the cellulosic ether and with BCP, the detection is available to 0.5 ppm (Fig. 2) but the results are non uniform on the film.
Mapping of the Ru distribution was performed and possible.

The labeling of the Si-polymer was verified in two conditions : 0.1 and 0.01 eq Ru/Si. The detection was maintained after dialyse and confirms that some amount of Ru was fixed to the polymer (Fig. 3).

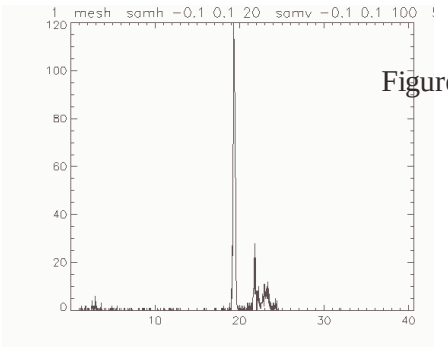
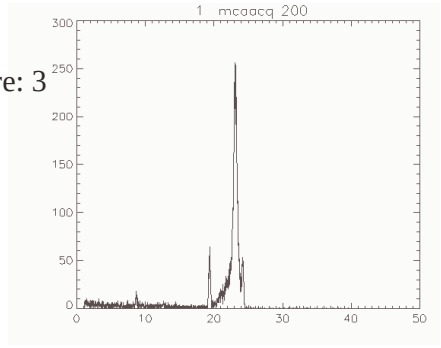


Figure: 3

4a2fmap_001 ; Labeling gel with 0.1 eq/si (film)



4b2f_005 ; Labeling gel with 0.01 eq/si (film)

We expected quantification of the Ru in the conditions described previously and spatial identification and concentration of the Ruthénium complex This quantification is not yet realized but it seems to be difficult because of the probably heterogenous labeling.

Conclusion

This is a preliminary conclusion wich suggests that the detection of Ru with this method is enough sensible to answer the question :
Is the reticulated polymer degradable in biological conditions?
Is it an enzymatic or a cell process?
Is the polymer stay in the surgical site under skin or in bone after bone ingrowth and biomaterial substitution?
Qualitatives or semi-quantitatives results will be sufficient to answer of these questions.