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Purification of biomimetic apatite-based hybrid colloids intended for biomedical applications: A dialysis study

Ahmed Al-kattan[a,b], Pascal Dufour[b], Christophe Drouet[a,*]

[a] CIRIMAT Carnot Institute–Phosphates, Pharmacotechnics, Biomaterials, University of Toulouse, CNRS/INPT/UPS, ENSIACET, 4 allée Emile Monso, 31030 Toulouse cedex 4, France
[b] CIRIMAT Carnot Institute, University of Toulouse, CNRS/INPT/UPS, LCMIE, Université Paul Sabatier, 118 route de Narbonne, Bât. 2R1, 31062 Toulouse cedex 9, France

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ABSTRACT

The field of nanobiotechnology has lately attracted much attention both from therapeutic and diagnosis viewpoints. Of particular relevance is the development of colloidal formulations of biocompatible nanoparticles capable of interacting with selected cells or tissues. In this context, the purification of such nanoparticle suspensions appears as a critical step as residues of unreacted species may jeopardize biological and medical outcomes, and sample purity is thus increasingly taken into account by regulatory committees. In the present work, we have investigated from a physico-chemical point of view the purification by dialysis of recently developed hybrid colloids based on biomimetic nanocrystalline apatites intended for interacting with cells. Both Eu-doped (2 mol.% relative to Ca) and Eu-free suspensions were studied. The follow-up of the dialysis process was carried out by way of FTIR, TEM, XRD, pH and conductivity measurements. Mathematical modelling of conductivity data was reported. The effects of a change in temperature (25 and 45 °C), dialysis medium, and starting colloid composition were evaluated and discussed. We show that the dialysis method is a well-adapted and cheap technique to purify such mineral–organic hybrid suspensions in view of biomedical applications, and we point out some of the characterization techniques that may prove helpful for following the evolution of the purification process with time.

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1. Introduction

During the last decade a lot of efforts have been dedicated to develop innovative nanosystems [1–5], often formulated as colloids, capable of interacting specifically with cells and tissues. A particularly developed domain of investigation involves cancer cells targeting. The idea is here to improve the efficacy of cancer treatments while significantly reducing side effects, and to facilitate early diagnosis therefore limiting cancer-related mortality. Indeed, according to the International Agency for Research on Cancer (CIRC), 24 million people are currently affected by cancer worldwide [6]. In its 2008 World cancer report, this Agency estimated that the number of patients concerned by this disease, originating from the anarchical proliferation of some cells, had doubled between the years 1970 and 2000.

In the field of cancer diagnosis in particular, medically oriented detection techniques based on luminescent nanoprobes capable of interacting with cells have particularly raised interest in the view of tumour detection. Among the nanoprobes commonly considered for medical imaging are organic dyes such as DAPI or green fluorescence protein [7] or semiconductor quantum dots [8–10]. However, these systems appear to be either toxic [11,12] or non-suitable for the analysis of biological tissues over extended periods of time due to photobleaching or flickering effects [13,14]. Taking account of these above observations, alternative nanosystems have been investigated, such as lanthanide-doped inorganic nanoparticles characterized by narrow emission bandwidths, high photochemical stability and long fluorescence lifetime (up to several milliseconds). Also, as for quantum dots, different colours are available by varying the luminescent centre used, e.g. Tb, Eu, Dy [15–18]. In this view, we have successfully synthesised recently by soft chemistry, stable luminescent colloidal systems based on biomimetic nanocrystalline apatite analogous to bone mineral [19], $Ca_{10-x}(PO_4)_{6-x}(HPO_4)_x(OH)_{2-x}$ $(0 \leq x \leq 2)$, doped with a few atomic % of rare-earth element [20].

In our previous work [20], the synthesis and physico-chemical characteristics of such colloids (size, morphology, composition and luminescence properties) were investigated and perspectives in terms of biological evaluations were commented. The synthesis protocol used for preparing such colloidal apatite nanoparticles involves the use of ionic salts, some of which being used in excess,

* Corresponding author. Tel.: +33 034 32 34 11; fax: +33 034 32 34 99.
E-mail address: christophe.drouet@ensiacet.fr (C. Drouet).

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and a purification process is then necessary for eliminating unreacted salts.

More generally, purification may appear as one of the limiting steps of development of potentially promising nanoscale systems and should not be under-estimated. In fact, it should probably be considered early in the process of developing innovative systems, as remaining traces of unreacted reagents, catalysts or organic solvents may lead to biased biological results or the impossibility to comply with regulatory aspects.

Two categories of procedures are often encountered (e.g. in pharmaceutical or agro-alimentary developments) for purifying suspensions.

A first approach consists in preliminarily separating the solid particles from the liquid medium by way of filtration (e.g. vacuum filtration or syringe filtering) or fast-drying methods (e.g. in fluidized-bed) and subsequently washing the obtained powder or gel with an appropriate pure solvent such as deionised water. However, this procedure is not straightforward as it requires both a separation and a washing step. Also, the filtration may appear in itself difficult in the case of nano-sized particles as they can often pass through the pores of filters, resulting in inefficient filtration, while smaller pores can rapidly be obstructed by accumulated particles leading to extremely low filtration yields. Finally, the possibility to re-suspend the particles at the end of the process often proves to be problematic due to some irreversible agglomeration of adjacent particles during the separation step.

The second approach aims at retaining the nanoparticles in a wet state throughout the whole cleansing process in order to avoid potential agglomeration issues upon particle drying. Centrifugation may be considered in this second type of approach. The aim of this technique is to activate the sedimentation of the particles by action of a centrifugal force. This particle settling effect enables then to exchange (generally at the occasion of many successive rounds) most of the supernatant with pure solvent, thus leading progressively to purified suspensions. However, regular centrifugation often shows limitations for the sedimentation of nano-sized particles, while the use of ultracentrifugation can only be dedicated to purify small volumes of suspensions and appears rather energy- and time-consuming.

Based on the above considerations, we have investigated here, from a physico-chemical viewpoint, the purification process of hybrid biomimetic apatite-based colloidal nanoparticles by way of dialysis (in aqueous conditions). Dialysis is indeed another method based on the second type of purification approach, during which the nanoparticles remain in liquid medium at all times. Dialysis is largely used in medicine for example for treating kidney diseases. Unlike ultrafiltration or reverse osmosis, dialysis does not need to apply a gradient pressure, which thus presents an obvious practical advantage, and remains a rather cheap method. It is based on the diffusion of molecules through a permeable membrane due to a concentration gradient [21] which is applied between both sides of the membrane: the dissolved substances (e.g. unreacted ions or un-adsorbed organic molecules) move from the high-concentration region (inside the membrane) to the low-concentration region (outside the membrane). Despite the use of dialysis for specific medical treatments, purification studies of nanoparticle-based systems are very scarce in the literature, and generally remain poorly documented from a physico-chemical viewpoint.

In the present contribution, we studied the dialysis purification of such apatite-based colloidal suspensions intended for biomedical applications, in the case of both Eu-doped and Eu-free systems. We report complementary physico-chemical data on the follow-up of the dialysis efficacy. Particular attention was dedicated to various physico-chemical parameters including conductivity, pH, temperature and the nature of dialysis medium.

2. Materials and methods

2.1. Preparation of apatite-based colloids

The hybrid mineral-organic apatite-based colloids prepared in this work were obtained by coprecipitation at room temperature of calcium and europium nitrates and ammonium hydrogenphosphate in deionised water, at pH 9.5, in the presence of a biocompatible stabilizing agent: 2-aminoethylphosphate or "AEP", responding to the formula $NH_3^+–CH_2–CH_2–O–P(O)(O^-)_2$. the precipitates were then allowed to age in an oven preset to 100 °C for 16 h. The AEP molecules represent the polar head of a natural phospholipid, phosphatidyl-ethanolamine, that is already present on the lipid bi-layer of human cells [22]. Our previous investigations indicated that such AEP^- molecules could strongly interact with surface calcium ions, thus exerting both an electro-steric repulsive effect preventing/limiting the agglomeration of adjacent nanoparticles, and thus providing colloidal stability to the nanocrystalline apatite suspensions [20,23,24]. Unless otherwise specified, for these colloidal suspensions the starting AEP/(Ca + Eu) molar ratio was set to 1. This value has indeed been shown [20,24] to lead to stable deagglomerated apatite particles with mean particle size around 30 nm.

For each preparation, three aqueous solutions were prepared: solution (A) contained a total of 4.87 mmol of calcium nitrate ($Ca(NO_3)_2{\cdot}4H_2O$) and europium nitrate ($Eu(NO_3)_3{\cdot}6H_2O$) with Eu/(Eu + Ca) molar ratio in the initial reaction mixture fixed at 1.5%. Solution (B) contained 4.87 mmol of AEP in deionised water. Finally, solution (C) was prepared from dissolving 1.62 mmol of ammonium hydrogenphosphate in deionised water, with an initial molar ratio (Ca + Eu)/P of 0.33 (ratio used previously for the preparation of apatite colloids in the presence of AEP [20,24]. Solution (A) was mixed with solution (B) under constant stirring. The acidic pH of the resulting solution, leading to solution (D), as well as that of solution (C) were adjusted to 9.5 by addition of ammonia. This alkaline pH was chosen in order to favour the precipitation of apatite rather close to stoichiometry exhibiting good chemical stability [20,24]. The pH of the colloids may be adjusted, after synthesis, to physiological value (7–7.6) in view of biological applications.

For comparative purposes, "reference" non-colloidal apatite suspensions were also synthesized, using a similar protocol as above but without addition of AEP. In this case, solution (B) contained only deionised water.

2.2. Dialysis protocol

In a typical dialysis procedure for this study, a tubular cellulose membrane (length: 15 cm, diameter: 3 cm, cut-off: 6000–8000 Da) was contacted with deionised water during 5 min for preliminary hydration. After clamping the lower end of the membrane, 25 ml of the suspension to dialyse were introduced and the second end was clamped while leaving an open space above the liquid level of about 7 ml filled with air. The membrane was suspended vertically by way of a fixing device and introduced in 800 ml of dialysis medium (e.g. deionised water). As mentioned in the text, the washing medium was continually homogenized by mechanical stirring, and was regularly exchanged by a fresh one (up to 3 days) so as to regenerate the concentration gradient and accelerate the purification process. Unless otherwise specified, the dialysis process was carried out at room temperature (25 °C). Some experiments, as indicated in the text, were run at 45 °C. In this case, the response of the conductivity electrode was then corrected for temperature effects based on preliminary calibration.

The principle of dialysis is shown on Fig. 1. During this process the dissolved unreacted species (Ca^{2+}, PO_4^{3-}, AEP^- and co-ions: NO_3^-, NH_4^+) moved by the concentration gradient, cross the pores

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