DALTON
FULL PAPER

Polymorphism and architectural crystal assembly of calcium carbonate in biologically inspired polymeric matrices†

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Received 26th April 2000, Accepted 7th July 2000
First published as an Advance Article on the web 26th September 2000

The control of the polymorphism and architectural crystal assembly of calcium carbonate minerals in gels formed by means of collagenous matrices with entrapped polypeptides is reported. It has been observed that the calcium carbonate polymorphic selectivity is related to the local supersaturation within the microenvironment where nucleation and growth occur. This crucial parameter is controlled in terms of the entrapped additive concentration and of the tailoring of the biopolymeric scaffold by mechanical deformation. Specific orientation effects and crystal aggregation of the mineral phases can be controlled either by the charged polypeptide with a beta structure or by the structural organization of the triple helical stretches in the collagenous matrix. This results in the growth and assembly of crystals into desired shapes and sizes by molecular recognition at a definite crystal face or by the control of the organic macromolecular microenvironment fit in the emerging area of biologically inspired approach to structured inorganic materials with appropriate physical and chemical properties.

Introduction

Biomineralization in living organisms leads to the formation of inorganic–organic composites. Although the content of the organic component in a typical composite is very low (of the order of 1 wt%), it exerts tremendous control on the mineralization process leading to particles of uniform size, novel crystal morphology, and specific crystallographic orientation.[1] In recent years many studies have been carried out on the composition and structure of the organic matrix, either in its water-soluble components or its insoluble ones.[2] It has been found that acidic macromolecules extracted from aragonitic or calcitic mollusk shell layers are responsible for the selective precipitation of aragonite and calcite *in vitro*.[3,4] On the basis of the available information several scientists have started to use biologically inspired systems to control the mineral deposition. The usual strategy has been to build a tailor-made matrix in which the mineral phase nucleates by an epitaxial mechanism.

Poly-L-aspartate has been considered an analogue of acidic macromolecules,[5] which are bound to an insoluble protein matrix with gelling properties. These properties could be related to the compartmental strategy used to control size, shape and aggregation of biological crystals.

The crystallization in gels has been thoroughly studied and gels have been used to control the diffusion process of the ions in the media.[6] We have used an approach in which the gel has an active role not only on ion transport but also in the modeling and organization of the mineral crystalline units. We showed that crosslinked gelatin xerogels[7] with entrapped polypeptides have favourable properties for the controlled crystallization of calcium carbonate in biologically inspired routes.[8] We have found that the concentration of the entrapped polyelectrolyte, its conformation, and the macromolecular orientation induced by uniaxial deformation of the xerogels control the formation of calcium carbonate polymorph crystals and their architectural assembly.

† Based on the presentation given at Dalton Discussion No. 3, 9–11th September 2000, University of Bologna, Italy.

Experimental

High purity $(NH_4)_2CO_3$, $CaCl_2{\cdot}2H_2O$ reagents manufactured from Merck Co., type A gelatin from porcine skin (300 Bloom), poly-L-aspartic acid (sodium salt; MW 9600) and poly-L-glutamic acid (sodium salt; MW 9600) from Sigma Co. were used. Deionized water (2 μS, Millipore) was used.

Gelatin xerogels containing polypeptides were obtained as previously described.[8] The cross-linked gelatin films become more and more brittle on increasing the entrapped polypeptide concentration. Therefore the highest used polypeptide concentration was 300 mg of polypeptide per gram of gelatin. At this concentration the films can be uniaxially deformed only up to 150% elongation. Calcium carbonate crystals were grown into the films at 18 °C by slow diffusion of $(NH_4)_2CO_3$ vapor in calcium chloride solution (10 mM), as described by Addadi and Weiner.[9] Crystallization solutions with different Mg^{2+}/Ca^{2+} molar ratios (0.0; 1.0; 2.5) were obtained by adding the required mass of magnesium chloride to a known volume of a 10 mM calcium chloride solution. Although it is difficult to reproduce the nucleation density, the same mineral forms were observed in repeated experiments (at least more than five). The mineral phase reported indicates the main phase precipitated as observed by X-ray diffraction and FTIR spectra. In some concentration ranges we clearly observed two calcium carbonate polymorphs. When this happened both phases were reported.

Morphological investigations were carried out using an optical microscope and a Philips XL-20 scanning electron microscope. High angle X-ray diffraction analysis employing Cu-Kα radiation was carried out by means of a flat camera. Fourier transform infrared spectra (FTIR) were obtained from KBr pellets using a Nicolet 250 FTIR spectrometer.

Results and discussion

Controlled crystallization of calcium carbonate minerals in collagenous matrices

We used collagenous matrices containing entrapped poly-L-aspartate (poly-Asp) or poly-L-glutamate (poly-Glu) as nucleating substrates of calcium carbonate polymorphs. Calcite,

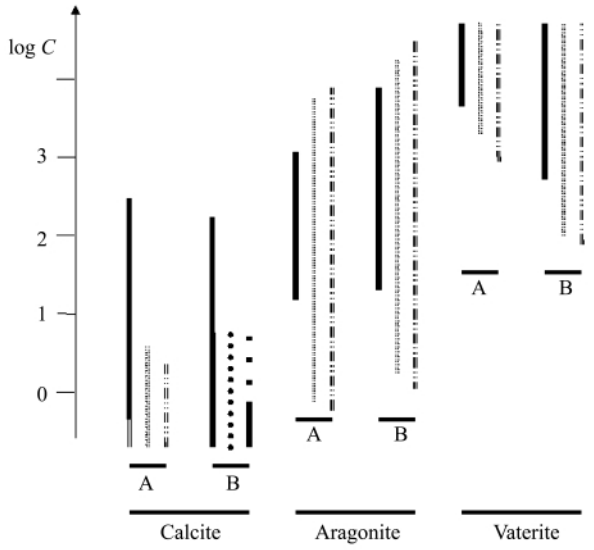


Fig. 1 Ranges of concentration in which the calcium carbonate polymorphs formed in the collagenous matrices. The concentration (*C*) is expressed in μg of polypeptide per gram of gelatin. (A) Collagenous films containing entrapped poly-L-aspartate. (B) Collagenous films containing entrapped poly-L-glutamate. The different lines indicate the degree of uniaxial deformation of the collagenous matrices. (▬): Unoriented collagenous films; (• • •): collagenous film elongated 100%; (▬ ▪): collagenous film elongated 150%. The single and double lines indicate the unoriented and oriented mineral phase respectively.

aragonite and vaterite were detected by X-ray diffraction and FTIR spectra.[10] Optical and scanning microscopy were used to localize the presence of calcite on the matrix surface and of aragonite and vaterite inside the matrix. The effect of polypeptide concentration and uniaxial deformation of the matrix are summarized in Fig. 1. The control over the polymorphism is a function of the amount of entrapped polypeptide in the collagenous xerogels and of the degree of uniaxial deformation. In the presence of unstretched collagenous matrices we can observe three ranges of concentration of poly-Asp where a calcium carbonate polymorph is the main or unique phase detected (Fig. 1). At concentration of entrapped poly-Asp less than 0.5 μg per gram of gelatin the (001)-oriented crystallization of calcite on the matrix surface was observed. An increase in the polypeptide concentration induces the formation of unoriented crystalline aggregates of calcite on the matrix surface. The presence of calcite was observed for a maximum concentration of poly-Asp of about 0.5 mg per gram of gelatin. At poly-Asp concentrations ranging from 0.5 mg to 10 mg per gram of gelatin, unoriented aragonite inside the collagenous matrix was observed. Unoriented vaterite was found at the highest concentration of poly-Asp used.

It is reported that poly-Glu is in a random coil conformation under our experimental conditions where the poly-Asp is in an ordered β sheet structure.[5] The influence of the charged polypeptide structure on the crystallization of calcium carbonates was evaluated by comparing the behaviour of poly-Asp with that of poly-Glu. The comparative results, reported in Fig. 1, show that in the presence of poly-Glu the ranges of polypeptide concentrations specific for the nucleation of a mineral and the crystals orientation respectively are different from those found in the presence of poly-Asp. In fact when we entrapped poly-Glu in the collagenous matrix calcite crystals were not oriented and the control over the polymorphism was reduced (Fig. 1). Furthermore aragonite and vaterite crystallized together in a large range of poly-Glu concentrations.

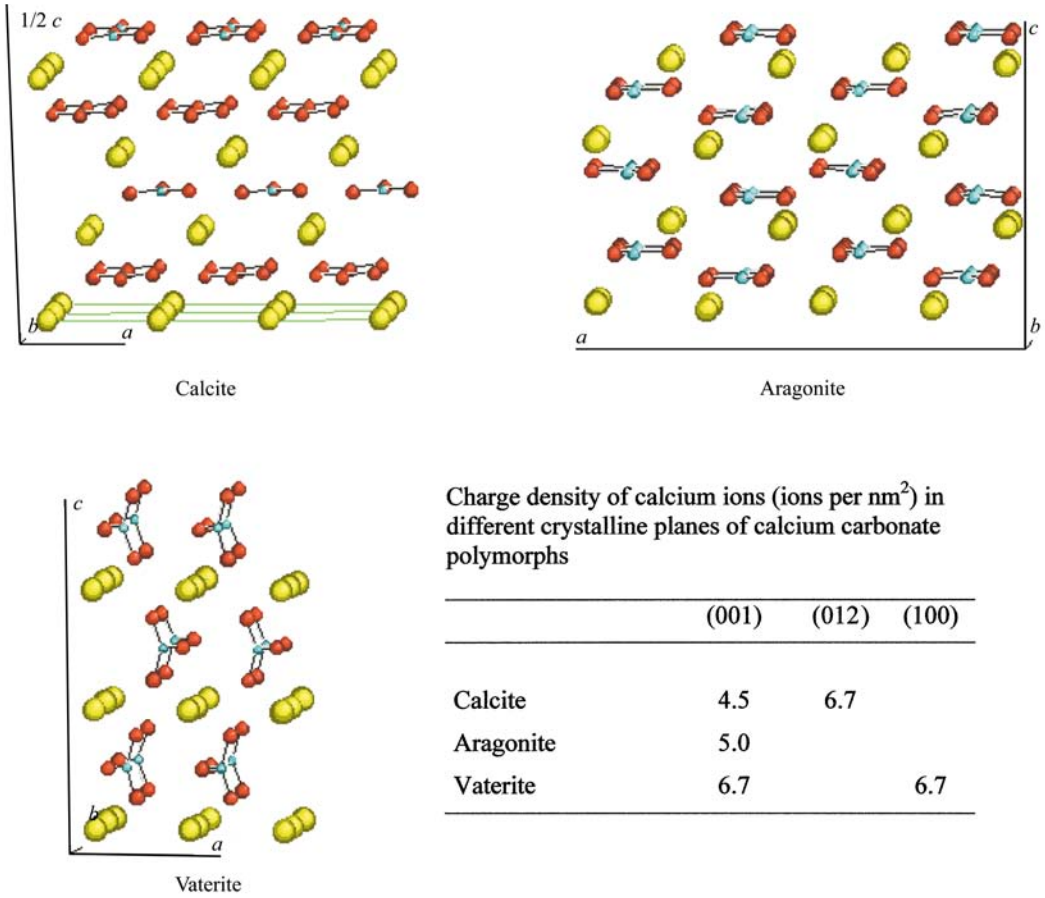
The uniaxial deformation of the xerogel provokes an orientation and reorganisation of the molecules. The X-ray data show that stretches of the collagen molecules are oriented with their helical axes parallel to the direction of deformation and the swelling data show that the average pore volume is reduced by stretching.[8b] The uniaxial deformation of the films influences the mineral deposition (Fig. 1). Increasing the uniaxial deformation increases the stability ranges of aragonite and vaterite, and reduces the calcitic one. There is a clear trend moving from 0% to 150% elongation through 100% elongation of the organic matrix (Fig. 1). Also the orientation of the mineral crystals deposited inside the collagenous xerogels depends on the uniaxial deformation. Indeed aragonite and vaterite crystals are preferentially oriented with a crystallographic axis parallel to the stretching direction of the xerogel. The effect of magnesium ions in solution on the crystallization of calcium carbonate polymorphs by means of collagenous matrices was also investigated. We found that magnesium does not influence the crystallization of aragonite and vaterite, but it allows a high level of isomorphic substitution of magnesium ions to calcium ions into the calcite structure.[8b]

The observed matrix effects on calcium carbonate polymorph precipitation can be explained invoking the Ostwald rule. This rule predicts that the initial mineral formed from a solution supersaturated with respect to more than one mineral is the one with the highest solubility, that is, in our case vaterite. In the xerogel the local supersaturation is a function of the negatively charged polypeptide concentration as well as of the volume of the pores where the nucleation occurs. On the xerogel surface there is no control on the volume of the nucleation sites, and the supersaturation is only related to the bonding of calcium ions to the negatively charged groups of the polypeptides. There the crystallization of calcite, the least soluble polymorph, is observed.

Inside the xerogel the decrease in the volume of the nucleation sites due to the uniaxial deformation, and the related increase in the negatively charged polypeptide concentration, leads to high local supersaturations. Thus the switch in the precipitation from aragonite to vaterite occurs in uniaxially deformed xerogels with a high concentration of entrapped negatively charged polypeptides. The stronger electric field due to the higher concentration and more closely spaced three-dimensional distribution of the negatively charged carboxylate groups should favor the interaction with the most positively charged crystalline plane. Vaterite should preferentially interact with the two homocharged calcium planes[11,12] (001) and (100) with a charge density of about 6.7 calcium ions per square nanometer, as well as calcite with (012) calcium planes[13] (charge density 6.7 calcium ions per nm^2), or with the (001) calcium planes having 4.5 calcium ions per nm^2 and aragonite with (001)[14] plane having 5.0 calcium ions per nm^2 (Fig. 2). These observations and the crystallographic orientation of the mineral phases in the uniaxially deformed matrices are in agreement with our model of oriented calcium carbonate crystallization in collagenous xerogels induced by interaction of the negative carboxylate groups of poly-Asp with one of the most positively charged crystal planes of calcite, aragonite and vaterite as shown in Fig. 3.

Architectural assembly of calcium carbonate minerals in collagenous matrices

The architectural assembly of the crystalline units of calcium carbonate minerals in collagenous xerogel can be controlled either by the mechanical deformation of the collagenous xerogel or by the presence of a polypeptide in an ordered conformation (poly-Asp). We have shown above that calcite crystals in the presence of poly-Asp grow with their *c*-axis perpendicular to the xerogel surface and that the crystalline units of aragonite and vaterite orient themselves either as a response to an epitaxial growth in the collagenous matrix containing poly-Asp or as a result of mechanical constraints induced by the organization of the collagenous fibers. These two mechanisms can both be active when uniaxially deformed xerogels containing poly-Asp are used. In Fig. 4 vaterite grown inside



Charge density of calcium ions (ions per nm^2) in different crystalline planes of calcium carbonate polymorphs

	(001)	(012)	(100)
Calcite	4.5	6.7	
Aragonite	5.0		
Vaterite	6.7		6.7

Fig. 2 Schematic representation of the crystal structure of calcite, aragonite and vaterite (the subcell of vaterite is shown with one of three possible orientations of carbonate ions[19]). Positively charged planes of the three polymorphs are reported in the table of the insert.

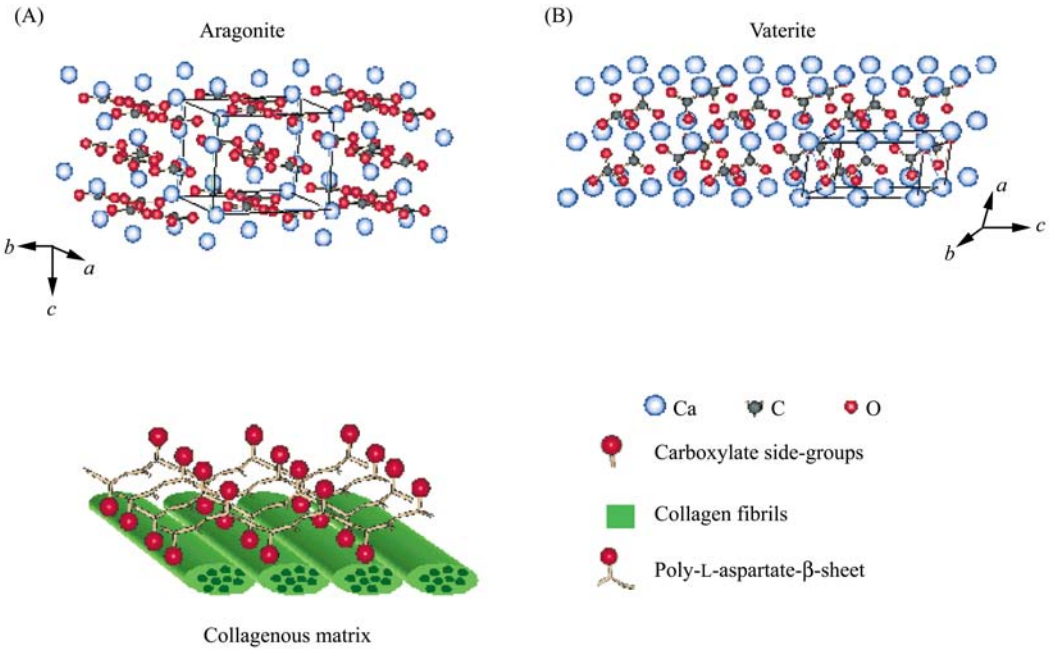


Fig. 3 Schematic model of the oriented nucleation of aragonite (A) and vaterite (B) in uniaxially deformed collagenous matrices containing poly-Asp. One or the other of the two polymorphs of calcium carbonate is induced by different amounts of entrapped polypeptide.

undeformed or uniaxially deformed xerogel containing the same concentration of entrapped poly-Glu is shown. We can observe the crystalline units form spherulites in the undeformed xerogels and align in the direction of uniaxial deformation in stretched xerogels. This control of the crystalline units assembly seems to be related only to the mechanical constraints due to the collagenous fiber organization. In fact the degree of orientation of the crystals is related to the degree of alignment of the collagenous fibers.

The presence of magnesium in solution induces a substantial change in the crystal morphology of the mineral phase precipitated.[15] In the first stage of the crystal growth, at magnesium/calcium molar ratios equal to 1 and in the absence of entrapped poly-Asp, magnesium calcite crystallizes as prisms elongated in

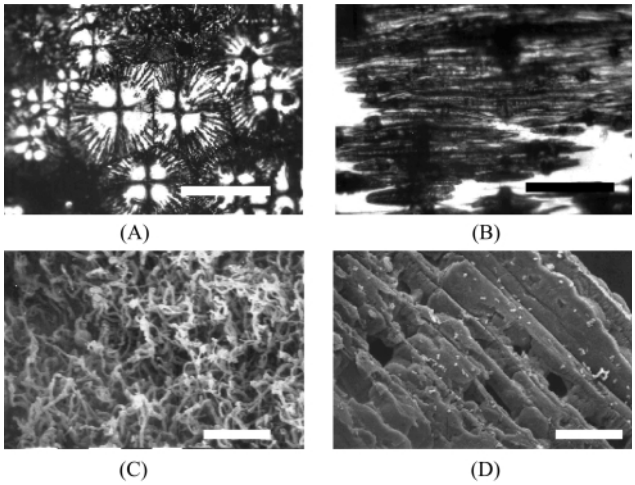


Fig. 4 Vaterite aggregates grown in unstretched (A and C) and uniaxially deformed (B and D) collagenous matrix with entrapped poly-Glu. (A) and (B) optical micrographs in cross polar. (C) and (D) scanning electron micrographs of deproteinated vaterite aggregates. Scale bar: 20 μm.

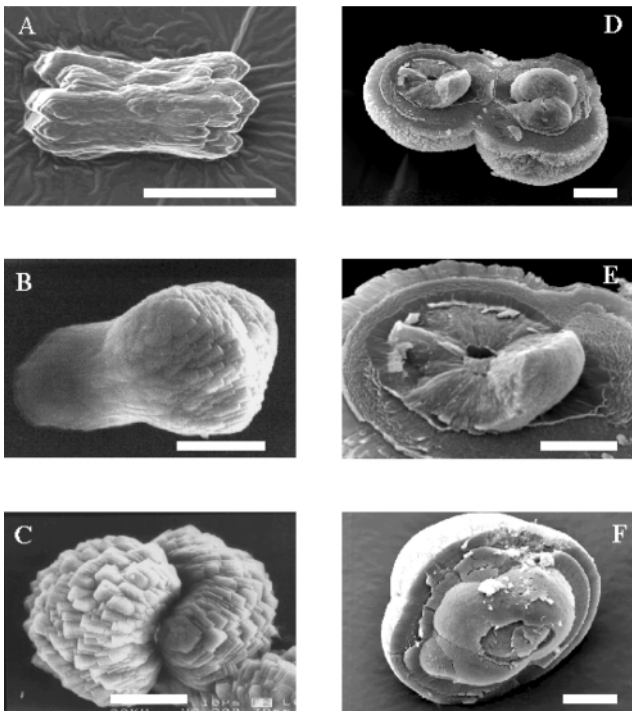


Fig. 5 SEM images of a globular aggregate of magnesium calcite crystals grown in the presence of a collagenous matrix without (A, B, C) and with (D, E, F) entrapped poly-Asp. A, B, and C show the morphological evolution of an aggregate as a function of the crystallization time. In D, E, F some representative views of "ooids" like aggregates are shown. Scale bar: 10 μm.

the direction of the *c*-axis and capped with rhombohedral (104) faces. These crystals aggregate with an angular spread within a few degrees of the hexagonal axes. During the growth the angular spread increases until the aggregate ends in a globular shape (Fig. 5). These stages of growth of magnesium calcite aggregates mimic the morphological evolution of magnesium calcite and fluoroapatite aggregates in silica gel and gelatin matrices respectively. Thus it seems likely that the gel released by the film surface dictates the aggregates morphology. In the presence of entrapped poly-Asp, in the range of concentration of calcite precipitation, the rhombohedral faces exhibit more rounded edges, which disappear completely at higher concentrations of entrapped polypeptide. At magnesium/calcium molar ratios of 2.5, the aggregates of acicular magnesium calcite crystal, elongated in the direction of the *c*-axis, consist of a smaller hemisphere grown inside the film joined with a larger one grown toward the solution. A central core surrounded by concentric shells (generally two) of radially oriented crystals forms the smaller hemisphere. The diameter of the inner hemisphere was about 40–60 μm and the average thickness of an external shell was about 1.5 μm (Fig. 5). The formation of these shells could be related to the compartmental structure of unstretched gelatin films. The adsorption of poly-Asp on the (001) plane of calcite may interrupt the crystal growth along the *c*-axis direction. The polymeric covering thus formed might serve as a substrate for further crystallization, giving rise to another shell, and so on with a mechanism similar to that proposed for the formation of the ooids from a supersaturated seawater solution containing humic acids.[16] The homogeneity of the shell thickness and the presence of a layered structure at different stages of growth may exclude any recrystallization effect. These concentric shells were not observed in the larger hemisphere, which consists of radially oriented acicular magnesium calcite crystals.

Addadi *et al.* observed similar concentric shells in magnesium calcite aggregates, formed from an amorphous phase, where the external shell has a different magnesium content with respect to the core.[17] Kniep *et al.* observed these core/shell assemblies for fluoroapatite grown in gelatin.[18] Many aspects of magnesium calcite aggregations in the presence of or inside gelatin xerogels are similar to those described for magnesium calcite or fluoroapatite grown in gels of silica[6b] or gelatin[18] respectively. This suggests that the morphological evolution during the growth of acicular crystals aggregates in the gels is mainly related to the gelling properties of the medium of crystallization (Fig. 5). These properties of the organic matrix might be also responsible for the architectural assembly of biogenic crystals in mineralized tissues and organisms. At the highest concentration of entrapped poly-Asp we have always observed the formation of aragonite and vaterite. In these cases the presence of magnesium, which does not substitute calcium in these crystal lattices, seems to affect only the morphology of the crystalline aggregates that appear with more rounded faces.

Conclusions

Collagenous xerogels are versatile systems in which the nature, density and structural geometry of the charged groups responsible for the crystallization of calcium carbonate can be easily regulated by changing the kind and the amount of acidic polyelectrolytes in the starting gelatin solution. The structure of the polyelectrolyte-gelatin assembly can be easily changed through mechanical deformation, which modifies the microenvironments of the nucleation and growth sites. These peculiar properties of the collagenous matrices are exploited to control calcium carbonate morphology, and magnesium calcite orientation and aggregation as a function of the amount and kind of negatively charged polypeptide present and/or of the degree of the film deformation. These studies of the assembly and growth of crystals into desired shapes and sizes by the control of the organic microenvironment in a xerogel fit in the emerging area of biologically inspired approach to structured inorganic materials with appropriate physical and technological properties.[1]

Acknowledgements

Financial support from the Consiglio Nazionale delle Ricerche, Ministero dell' Universita' e della Ricerca Scientifica and University of Bologna (Funds for selected research topics) is gratefully acknowledged.

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