Biomimetic structures: polymeric and supramolecular scaffolds for controlled cell growth and CaCo3 crystallization

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BIOMIMETIC STRUCTURES
Polymeric and Supramolecular Scaffolds for
Controlled Cell Growth and CaCO₃ Crystallization

PROEFSCHRIFT

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Chapter 1

Biomimetic structures

Abstract

Elucidating the basic building principles of living organisms offers the possibility to design innovative bio-inspired materials with yet unrealized properties, which may be used for different biomedical applications. This chapter introduces the concept of biomimetic structures and illustrates it with examples from the literature. The first part presents in vitro 2D model systems, which have been employed to achieve a better understanding of the processes involved in the formation, functioning and regeneration of tissues such as muscle and bone. Of particular interest in this respect are patterned surfaces that can be used to direct the organization of living cells in vitro. The second part describes the progress made in understanding the principles of biomineralization in order to achieve control over crystallization in vitro. Additionally, it discusses the aspects concerning the mechanisms of control of crystal nucleation and growth that still need to be resolved.
1.1 Introduction

Biomimetics or biomimicry (from bios, meaning life, and mimesis, meaning to imitate) refers to studying Nature and then imitating or taking inspiration from Nature’s building principles and processes to find solutions to human problems. To this end, biomimetics (or “innovation inspired by Nature” [1]) is currently being used to explore a variety of design projects, ranging from the development of different biomaterials, to solar cells inspired by the process of photosynthesis in plants, water-repellent coatings based on the lotus flower effect, artificial neural networks and robots based on animal models. In particular, the field of biomaterials represents one of the major applications of biomimetics, since the materials found in Nature reveal a high degree of organization with elaborate control over the structure, shape, size, orientation and assembly of the constituents, resulting in remarkable properties specifically selected for various functions. [2]

Biomimetics is a multi-disciplinary subject involving a wide diversity of domains like electronics, informatics, medicine, biology, chemistry, physics, and mathematics. The term of biomimetic structure or system refers to various aspects and involves imitation of Nature at different levels. As an example, in the field of biomedical applications (a relevant target of this thesis), biomimetic systems comprise:
- tissue or organ replacements, which mimic the original function closely
- biomaterials (e.g. for tissue engineering and drug delivery systems) that have the ability to interact with the biological environment by mimicking the biological recognition processes between cell receptors and biological molecules [3-7]
- organic-inorganic composites which try to mimic the high level of organization of biominerals at both the molecular and macroscopic level [8]
- *in vitro* patterned cell cultures that imitate the organization of tissues such as nerve and muscle [9]
- systems which mimic natural processes (such as the bone remodeling process)

This thesis focuses on three biomimetic systems, which are introduced in the next sections of the present chapter. These are: (a) *in vitro* patterned cell cultures as 2D models for muscle, (b) *in vitro* model systems for bone cell-biomaterial interaction and (c) 2D organic templates for controlled CaCO₃ crystallization.

1.2 *In vitro* model systems for soft and hard tissue

*In vitro* model systems have been used to achieve a better understanding of the processes involved in the development of hard and soft tissue as well as to study the interaction of the cells with scaffold materials. [2, 10] Model systems facilitate the study of chemical and physical properties at the interfaces between cells and the biomaterial or between organic and inorganic components (in biominerals, for example). This information can subsequently be used to design new bioinspired materials with superior properties (such as biocompatibility, mechanical properties, biomolecular recognition by cells etc.) as scaffolds for tissue
engineering. In addition, model systems are useful for the study of disease pathogenesis and for the development of molecular therapeutics. A very important issue when building a model system is the need to control the chemistry and organization of the material at the cell-substrate interface, as these aspects play a key role in modulating cellular behavior.

### 1.2.1 2D Patterns of cells

The possibilities for obtaining 2D patterns of cells *in vitro* have been broadly investigated, as the ability to direct the spatial arrangement of anchorage-dependant cells is important for fundamental studies of cell biology, biosensors and tissue engineering. In particular, achieving control of cell alignment is crucial to create organized structures such as nerve and muscle.

At present, there are two main approaches employed to direct cell attachment and orientation *in vitro*. The first approach relies on the use of topographical features to control cell alignment. It has been shown that different types of cells (e.g. osteoblasts, fibroblasts, neuronal and muscle cells etc.) adhering to microgrooved substrates that are long and narrow with respect to the length scale of a cell, align their bodies and cytoskeletons in the direction parallel to the length of the grooves. The alignment is influenced by the height of the microfabricated grooves, i.e. the extent of alignment increases with increasing the height of the features. These microgrooved substrates have been obtained on a variety of substrates, including polystyrene, acrylic polymers, collagen, titanium, as well as poly(lactic acid).

The second approach is the most commonly used and involves substrates patterned into regions with different chemistries. To control the spatial adhesion and orientation of the cells, areas that allow cell attachment (cell adhesive regions) are created next to cell inert (cell non-adhesive) regions. Extracellular matrix (ECM) proteins, such as fibronectin and laminin, from cell culture media are adsorbed selectively on the adhesive islands, promoting cell attachment. The non-adhesive regions prevent protein adsorption and thus cell attachment. In some cases, cell attachment and spreading was controlled by functionalization of the cell adhesive surfaces with the tripeptide sequence RGD and other similar peptide sequences, recognized by the cell membrane receptors. Examples of surfaces that prevent protein adsorption and cell adhesion include: OEG [oligo(ethylene glycol)] and PEG [poly(ethylene glycol)] - substituted alkanethiol self-assembled monolayers (SAMs), methacrylate-based comb polymers with pendant OEG side chains, mannitol groups, interpenetrating polymer networks of polyacrylamide and PEG, polyelectrolyte multilayer surfaces composed of poly(acrylic acid) and polyacrylamide, sulfonate-terminated alkylsilane SAMs etc.

Patterns of SAMs of alkanethiols on gold, generated by microcontact printing (µCP), provide a widely-used system to control the attachment and orientation of different types of cells (Figure 1.1 A). The advantage of this system is that a variety of functional groups and molecules can be introduced (e.g. CH₃, OH, NH₂, COOH, OEG, PEG, peptide sequences that bind to cell membrane receptors, etc.) – either before or after the monolayer is formed – to tune the properties of the monolayer surface. Cell adhesion and orientation was also controlled using (non-adhesive) perfluorinated alkylsilane SAM patterns on an (adhesive) aminoalkylsilane SAM background, obtained by exploiting the photochemistry of
organosilanes. Nevertheless, the application of SAMs is limited to a small number of substrates (e.g. Au, Ag, glass, silicon wafers) and the basic microfabrication techniques employed are often unfamiliar to biological laboratories.

As an alternative to SAMs, lift-off elastomeric membranes were used to pattern endothelial cells on glass, PDMS, polyurethane and silicon. Several groups have also micropatterned polymers to control cell-substrate interactions and some examples are presented below. Photosensitized copolymers of the thermoresponsive poly(N-isopropylacrylamide) were used to obtain patterns of fibroblasts on polystyrene (PS), exploiting the change in the wettability of the copolymer-grafted PS surface by varying the temperature between 37 ºC (hydrophobic – cell adhesive) and 10 ºC (hydrophilic – cell non-adhesive). Photoreactive phenylazido-derivatized copolymers have also been developed to control cell attachment on photochemically microprocessed surfaces. Cell patterning and alignment on biodegradable polymer substrates was achieved as well. µCP was employed to pattern lanes of laminin onto non-adhesive (BSA-coated) biodegradable PLGA (Figure 1.1 B) and elastomeric polyurethane films. In addition, this technique was used to deposit lanes of random copolymers of oligo(ethylene glycol) methacrylate and methacrylic acid on chitosan biodegradable substrates, making use of acid-base/electrostatic interaction. Also, a microfluidic patterning technique was developed to generate micron-scale patterns of any biotinylated ligand on the surface of a biodegradable polylactide-PEG copolymer. More recently, a patterning method based on µCP of a methacrylate-based comb polymer containing pendant OEG groups was reported. This method proved to be efficient for long term, spatially resolved attachment and growth of mammalian cells on different substrates (e.g. PS, PMMA, PET, gold). Furthermore, a polymer-on-polymer stamping (POPS) soft lithography technique has been developed, making use of electrostatic and other secondary interactions between polyelectrolytes. In this way, patterns of cell resistant multilayer films composed of poly(acrylic acid) and polyacrylamide were stamped on poly(allylamine hydrochloride), which was subsequently functionalized with RGD adhesion ligands. The advantage of this method is that it allows patterning of multilayers with variable functionalities on a variety of substrates.

In order to obtain model systems to study the interaction between different types of cells, several methods for the patterning of cell co-cultures have been developed. Bahtia et al. have patterned hepatocytes and fibroblasts using photolithography and manipulation of serum content of cell culture media. In addition, 3D microfluidic systems were employed to achieve patterning of different types of cells. In another approach electroactive SAM substrates were used to pattern two fibroblast populations by electrochemical switching of the substrate cell adhesiveness. More recently, Co et al. have reported a new system that allows patterning of different cell types on biodegradable chitosan and gelatin substrates through multilayer assembly of cell-resistant and cell-adhesive polyelectrolytes.
Chapter 1

Figure 1.1 (A) Schematic representation of the fabrication of alkanethiol SAM patterns by µCP: (a) preparation of the PDMS stamp and (b) pattern transfer by µCP. (B) Fluorescence microscopy image showing a cardiomyocyte pattern on PLGA membranes. Inset: pattern of laminin lanes conjugated to Oregon Green 488 underlying the cells. Reproduced from reference [18] (A) and reference [45] (B).

However, with some exceptions, most of the cell patterning techniques available at present either restrict the type of substrates that can be successfully patterned with cells or lack reliable, long term retention of cellular patterns under physiological conditions. Since substrate flexibility/rigidity can have a significant influence on cell differentiation, [54] it is also important to have the possibility to use polymer substrates with tunable flexibility. Moreover, organic surfaces with controlled chemistry, which allow functionalization with a wide range of groups and whose surface properties can be switched by external stimuli (such as temperature, pH or electric charge) are highly desirable. [2, 13] For that reason, conducting polymers represent an attractive class of materials and their application as substrates for cell culture is further detailed in Chapter 4.

1.2.2 2D Models for skeletal muscle

The mechanisms accompanying skeletal muscle tissue degeneration under mechanical loading (in the form of pressure sores, for example) are not completely understood. [55] In vitro model systems for muscle would be useful to study the effects of compressive straining on muscle cells, but also to determine the effect of for example electrical stimulation in the treatment of damaged muscle tissue. [56]

Mature skeletal muscle fibers consist mainly of fused, highly aligned, differentiated cells (Figure 1.2). The alignment of muscle cells is essential for effective muscle contraction and functioning in vivo. The extracellular protein matrix (ECM) plays an essential role in the alignment and differentiation of myoblasts (mononucleated precursor cells) and directed mechanical tension helps organizing the myoblasts into functional, aligned myotubes (multinucleated differentiated cells). In vitro however, in the absence of these guidance signals, cultured myoblasts are randomly oriented and, after differentiation into myotubes, the fibers lack an ordered structure. [57-59]
Figure 1.2 (A) Muscle formation. The process begins with muscle cell precursors becoming programmed to form muscle, at which point they are called myoblasts. The myoblasts then proliferate and migrate to the site of future muscle formation, where they fuse to produce myotubes. As myotubes mature, the sarcomere develops; this array of myosin and actin fibres forms the contractile unit of muscle. Reproduced from reference [60]. (B) Schematic view of a muscle fiber. Reproduced from reference [43].

Control of alignment of muscle cells (e.g. skeletal myocytes, cardiomyocytes, vascular smooth muscle cells) has been achieved in vitro using the two approaches illustrated in Section 1.2.1, namely grooved substrates [19, 20, 62, 63] and chemically patterned surfaces. [31, 45, 46, 58, 64] In addition, by applying a mechanical stretch, muscle cells can also be induced to orient themselves parallel to the direction of the force. [57, 65] Although the cell patterning methods described in Section 1.2.1 could in principle be applied to most types of mammalian cells, relatively few systems have been used to control the attachment and orientation of skeletal muscle cells. [19, 31, 58, 62, 64, 66]

1.2.3 Models for bone cell-biomaterial interaction

Bone is a highly organized composite material, consisting mainly of collagen – a protein-based hydrogel template – and inorganic apatite crystals with varying compositions and microstructures. Although bone has the ability to regenerate itself, this ability decreases with age, being also affected by diseases. The increased ageing of the population has generated higher demands for bone replacement materials. The materials that are currently used in the fabrication of orthopedic implants generally consist of a single component (e.g. metals, ceramics or polymers) or of a relatively coarse combination of two or three components. [6, 67-69] However, the performance of these materials in terms of mechanical properties and communication with the cellular environment is often not satisfactory. Therefore, there is a great interest in developing new bioactive materials (i.e. that can induce specific cellular responses and facilitate the integration of the implanted material in the surrounding bone tissue), with improved mechanical properties. [4, 6, 70-72] These materials should also have a controlled surface chemistry and topography, as these factors are known to significantly influence bone cell adhesion and differentiation. [73]
Figure 1.3 (A-D) Scanning electron micrographs of biogenic inorganic structures. (A, B) Silicic skeleton of unicellular organisms: radiolarian (A) and diatoms (B). (C) Part of the skeleton of a brittle star Ophiocoma wendtii (the mesh and the array of microlenses are composed of a single calcite crystal). (D) Shell nacre (calcium carbonate) of the primitive mollusk Neopilina hyalina. Scale bars: 10 µm (A, D), 1 µm (B) and 100 µm (C). (E) Schematic illustration of the organization of the nacre of the abalone shell. Reproduced from references [2] (A, B), [74] (C), [75] (D) and [76] (E).

Mineralized biological materials (e.g. bone, shells, diatoms) have elaborate 3D designs with exceptional microstructural, optical and mechanical properties (Figure 1.3 A-D). Inspired by Nature, several groups have prepared laminated composite films mimicking the seashell nacre (Figure 1.3 E), through directed nucleation and growth of inorganic materials such as calcium carbonate, calcium phosphate or silica on self-assembled organic templates. More examples of polymer-CaCO₃ composites can be found in Chapters 5 and 8.

In addition, synthetic bonelike apatite composites have been obtained by precipitation of calcium phosphates in the presence of surfactants, polyelectrolytes, biodegradable polymers, porous polymer matrixes, or dendrimers. Microemulsions were also used as pre-organized systems for the fabrication of crystalline calcium phosphate materials with elaborate 3D microskeletal architectures (Figure 1.4 A) or porous hollow shells of calcium carbonates with a honeycombed surface texture (Figure 1.4 B). It should be mentioned that the materials presented above represent only a few examples from the multitude of biomimetic inorganic-organic composite materials developed until now.

Bone cell – substrate interactions play an important role in the integration of the biomimetic scaffolds into the natural bone tissue. Therefore, in vitro model systems that facilitate the study of the interaction of bone cells with the biomaterial surface (e.g. cell attachment, proliferation, migration and differentiation) can provide valuable information for the design of new biomaterials with a controlled bone-biomaterial interface. Many groups have developed model systems to study in vitro the behavior of osteogenic cells on a wide variety of materials, such as calcium phosphate-coated metal substrates, calcium phosphate and calcium carbonate cements, biodegradable polymers, organic-inorganic composite materials, etc.
However, most of these systems do not allow live observation of the cells (i.e. by optical microscopy) during different stages of their development. Also, even if chemically or topographically patterned surfaces have been used to control the attachment and alignment of osteoblasts in vitro, \cite{101-105} the behavior of bone cells on patterned mineral substrates has not been investigated yet. 2D patterned substrates consisting of alternating mineralized and non-mineralized regions (which can be regarded as a 2D section of a porous 3D mineralized scaffold for bone tissue engineering) would also offer the possibility to compare the behavior of the cells on regions with different concentrations of mineral ions (such as Ca\(^{2+}\)). It is known for example that the extracellular calcium concentration controls osteoblast proliferation, differentiation and function. \cite{106, 107} and it has been suggested that the gradual dissolution of biomaterials such as hydroxyapatite, releasing Ca\(^{2+}\) ions, might be responsible for their osteoconductive properties (i.e. they stimulate the activity of osteoblasts, inducing new bone formation). \cite{106, 108}

### 1.3 Calcium carbonate

Calcium carbonate (CaCO\(_3\)) is one of the most abundant biominerals and has therefore received considerable interest in several areas of materials research. \cite{8, 77} In particular, CaCO\(_3\) has emerged as a promising material for bone replacement applications. \cite{67} Biogenic calcium carbonate (e.g. from coral skeletons and sea urchin spines) has been successfully used for bone replacement \cite{67} and nacre (present in mollusk shells) in particular showed remarkable osteoconductive properties. \cite{109}

CaCO\(_3\) exists in a variety of polymorphs, of which calcite and aragonite (with similar thermodynamic stabilities) are the most encountered in biological samples (Figure 1.5 A, B). Vaterite is metastable and extremely rare in nature (Figure 1.5 C). \cite{78, 110} Amorphous CaCO\(_3\) (ACC) is used by many organisms as a transient intermediate for the production of single crystals with elaborate shapes and sometimes it is present in combination with crystalline phases. \cite{111-113}
Figure 1.5 Typical morphologies (top) and unit cells (bottom) of (A) calcite, (B) aragonite and (C) vaterite. Calcite crystallizes in the hexagonal space group $R3c$ with lattice constants $a = b = 4.99 \, \text{Å}$ and $c = 17.06 \, \text{Å}$, aragonite in the orthorhombic $Pmcn$ group with lattice constants $a = 4.96 \, \text{Å}$, $b = 7.96 \, \text{Å}$, $c = 5.74 \, \text{Å}$ and vaterite in the hexagonal space group $P6_3$ with lattice constants $a = b = 4.13 \, \text{Å}$ and $c = 8.48 \, \text{Å}$. Reproduced from reference [114].

For biologically mineralized materials, the way in which the mineral and the organic material are organized are the key factors determining their unique properties. [75] Therefore, the comprehension of the way in which polymorph selection, as well as crystal shape and orientation are controlled in biomineralization would offer new opportunities for the design of novel biomimetic organic-inorganic composites for biomedical applications. Indeed, many research groups have investigated the ways to control the polymorph and the orientation of CaCO$_3$ crystals obtained \textit{in vitro}, as these factors have a strong influence on the ultimate material properties such as solubility (biodegradability) and mechanical properties.

1.3.1 Control of CaCO$_3$ formation in biomineralization

The study of biomineralization has a long history [115] and in the last decades significant progress has been made in understanding the principles underlying biomineralization processes. Nevertheless, the mechanisms of control of crystal nucleation and growth in biomineralization are not completely elucidated. [76, 78]

In Nature, several key steps regulate crystallization of CaCO$_3$: space confinement (i.e. the formation of a compartment, sealed off from the environment, in which mineralization takes place), the creation of an organized array of functional groups (the nucleation site), oriented nucleation, control of crystal growth and termination. [110, 116] The formation of biominerals is governed by the cooperation of soluble (usually acidic) and insoluble (hydrophobic, often cross-linked) macromolecules. The soluble macromolecules are rich in carboxylate groups and in some cases phosphate and/or sulfate groups. They form an intimate composite with the mineral phase at different hierarchical levels and are believed to play a significant role in control of crystal nucleation and growth. [75, 78, 110] For calcium carbonate-containing biological materials, these control macromolecules can be roughly divided into three categories: (1)
aspartic acid-rich proteins and glycoproteins, (2) polysaccharide-rich macromolecules and (3) glutamic acid- and serine-rich glycoproteins. The first two classes are in general associated with the crystalline mineral phases, while the proteins in the third class are commonly found to be associated with amorphous CaCO$_3$. The insoluble macromolecules constitute a structural 3D framework, which defines the space where the mineral phase forms. Examples of framework molecules include: glycine- and alanine-rich proteins (similar to silk-fibroin) in mollusk shells, $\alpha$-chitin in crustaceans and $\beta$-chitin in mollusk shells. Several studies have shown that some soluble acidic proteins are adsorbed onto the insoluble matrix. These molecules adopt specific $\beta$-sheet conformations, resulting in the formation of an organized array of acidic functional groups, which defines the orientation of the crystals (Figure 1.3 E). It has been suggested that oriented nucleation of crystals is also dependent on the degree of organization of the organic matrix.

At present, there are mainly three models proposed to explain the organic matrix-mediated oriented nucleation in biomineralization, a concept first elaborated by Lowenstam in 1981. The classical view suggests that there exists a structural and geometrical match between the lattice spacings in specific crystal faces and the organized array of charged functional groups present at the surface of the macromolecular matrix. The complementary inorganic cations bind at these sites forming a pre-organized layer of ions, followed by the binding of counteranions and cluster formation. Oriented nucleation is the result of the lowering of the activation energy for a specific crystallographic face (Figure 1.6 – pathway A).

Another possible mechanism involves matrix binding of crystalline nuclei formed either directly in solution or via the crystallization of amorphous clusters formed in solution. In this case the crystallographic orientation is determined by collective interactions between crystal surfaces of the preformed nuclei and the array of charged groups of the organic matrix (Figure 1.6 – pathway B). A more complex mechanism proposes that amorphous particles are formed by ion or cluster binding at the organic surface, followed by a matrix-mediated phase transformation, which results in oriented crystallization (Figure 1.6 – pathway C). In this
Chapter 1

model, oriented nucleation is the outcome of cooperative processes involving phase transformations, as well as molecular interactions similar to the ones described in the classical model of organic-matrix mediated epitaxy. It is important to note that presently the organic matrix is no longer seen as a static, rigid template, but as a dynamic interface that can adjust during the crystallization process. [120, 123]

1.3.2 Control of CaCO₃ crystallization in vitro

Inspired by biomineralization, different simplified molecular assemblies, exposing organized arrays of functional groups, have been used as nucleation templates to control CaCO₃ crystal orientation and polymorph specificity, including: Langmuir monolayers – compressed [124-133] or self-assembled monolayers (SAMs), [74, 142-152] functionalized gold colloids, [153] biological macromolecules, [118, 154] synthetic polymers, [155-157] surfactant aggregates [158, 159] etc. Other studies explored solution deposition of CaCO₃ in the presence of growth modifiers such as ions, proteins, [117, 160-165] (poly)peptides, [119, 166-168] synthetic polymers [169-177] or functionalized gold nanoparticles [178], which induced the selective formation of different polymorphs of CaCO₃ (e.g. calcite, aragonite and vaterite) or the stabilization of ACC.

Several studies have focused on systems that mimic the cooperative interaction between an insoluble organic matrix (e.g. SAMs, [179-182] Langmuir monolayers, [123, 183] collagen, [184, 185] sulfonated polystyrene, [186] polysaccharides [80] and soluble macromolecules [e.g. poly(acrylic acid), [80, 123, 180, 183] poly(aspartic acid), [184-186] poly(glutamic acid), [184, 185] polyglycerol [179]] or inorganic ions (such as Mg²⁺ [181, 182]) with the aim to control crystallization of CaCO₃ crystals and films. It has been shown, for example, that Mg²⁺ additives significantly modified the morphology of (01.2) oriented calcite crystals nucleated on carboxylic acid functionalized SAMs (Figure 1.7).

![Figure 1.7](image)

**Figure 1.7** Effect of Mg²⁺ ions on oriented growth of calcite on carboxylate-terminated alkanethiol SAM with an even number of methylene groups in the chain. Concentration Mg²⁺ (mol Mg²⁺/mol Ca²⁺) = 0 (A), 0.5 (B) and 2.0 (C). The insets present computer simulations of the morphologies of the corresponding calcite crystals nucleated from the (01.2) plane. In (A) the relative orientations of the SAM and the calcite crystal face it nucleates is also shown (bottom right). Scale bars: 50 μm (A) and 10 μm (B). Reproduced from reference [182].

In addition, Aizenberg et al. have used functionalized SAMs of alkanethiols, micropatterned by soft-lithography techniques, as spatially constrained, chemically modified
Biomimetic structures

microenvironments for crystallization, which offered the possibility to control the localization of the crystals, nucleation density, crystal size, orientation and morphology. CaCO₃ crystallization resulted in the formation of high-resolution inorganic replicas of the underlying organic patterns. Localized crystallization in these systems has been explained in terms of diffusion-limited, island-specific nucleation (for details see Chapter 5).

Although so many different biomimetic systems have been used to control CaCO₃ crystallization in vitro, the mechanisms responsible for oriented nucleation from an organic template are still under discussion. There are several views concerning the factors that determine orientation of the crystals nucleated from a template exposing an organized array of functional groups. Geometrical lattice matching, electrostatic interactions, template adaptability, as well as orientational complementarity between the template functional groups and the carbonate groups at the template/crystal interface have been considered key elements in determining crystal orientation.

Several researchers underlined the importance of epitaxy for achieving oriented nucleation of CaCO₃. Epitaxy involves a high degree of geometrical and orientational lattice matching between the pre-positioned array of functional groups of the template and the arrangement of calcium and carbonate ions in a specific crystal face. In many studies it was suggested that oriented nucleation of calcite or aragonite was the result of an epitaxial lattice match, i.e. the spacings of the acidic groups of the template were similar to the Ca-Ca distances and the orientation of the carboxylate head groups mimicked that of the carbonate anions in the nucleating crystal face.

There are also several reports in which the resulting orientation of the calcite crystals was related to a partial epitaxial match (i.e. geometrical match in one direction between the spacing of the functional groups of the template and one lattice distance in the nucleating crystal face) together with an orientation complementarity between the alignment of the functional groups of the template and the positions of the carbonate ions in the plane of nucleation (Figure 1.8 A). With some systems, however, no obvious correspondence was found between the spacings of the ions in the nucleating crystal plane and the spacings

![Figure 1.8 Example of (A) a both partial epitaxial and orientational match and (B) an exclusively orientational match between the carboxylate groups of the template and the carbonate ions in the nucleated calcite face.](image)

(a) SEM of oriented crystals; (b) Chemical structures of the molecules used as templates; (c) Model of the organic template (top) in relationship with the nucleated calcite face (bottom). Reproduced from reference [134] (A) and reference [157] (B).
between the functional groups of the template. In those cases, the formation of specific crystal faces was exclusively attributed to a match between the orientation of the carboxylate groups of the template and the carbonate ions in the nucleating face (Figure 1.8 B). [145, 147, 157, 187]

Volkmer et al. have suggested that non-specific electrostatic interactions played the most significant role in controlling the orientation of calcite crystals. [137-140, 168] Molecular dynamics simulations performed by Harding et al. also emphasized the importance of electrostatics. [188, 189] However, other factors, such as the supersaturation level of the mineralization solution, and thus the kinetics of CaCO₃ precipitation, were also found to have an additional influence on the morphology and the polymorph of CaCO₃ crystals. [129, 132, 133, 183, 190]

Oriented nucleation of vaterite crystals under octadecylamine and stearic acid monolayers was explained by a combination of electrostatic interactions and orientation matching between the crystal surfaces nucleated under the film and the alignment of the carboxylates (in the case of stearic acid monolayer) or the HCO₃⁻ ions bound to the monolayer (in the case of octadecylamine monolayer). Also in this case no geometric lattice matching between the monolayer and the nucleated crystal face was apparent. [124-126, 128, 129, 133]

Litvin et al. reported the formation of oriented aragonite crystals under self-organized Langmuir monolayer templates as the result of an epitaxial match between the orthorhombic aragonite and the rectangular lattice of the monolayer. [131] Later, Tremel et al. showed that the phase selection of aragonite on SAMs strongly depended on surface roughness. Aragonite formation was favored on poorly crystallized monolayer surfaces with a high roughness, whereas on highly ordered SAMs of functionalized alkanethiols with hexagonal symmetry the nucleation of calcite was favored. [142, 151, 152, 179] Tremel et al. also demonstrated that oriented nucleation of aragonite could be achieved on SAMs with a rectangular lattice packing, such as SAMs of anthracene terminated thiols. [142]

Surprisingly, although the importance of the flexibility of organic surfaces used as templates for CaCO₃ crystallization had been emphasized by Mann et al. already in 1988, [125] the ability of the template to adapt to the growing crystals was only rarely taken into account. [123, 140, 155, 188, 191] Charych et al. demonstrated the template adaptability for the first time in situ. [155] Polymeric films of 10,12-pentacosadiyonic acid were shown to reorganize upon calcite mineralization, in order to optimize the orientation fit between the carboxylates of the film and the carbonate ions in the (01.2) calcite face, as indicated by in situ FTIR and visualized by color changes in the polymer film. In many cases it was observed, for example, that more efficient control of crystal orientation was obtained under monolayers compressed at a low surface pressure, in which the molecules had a higher degree of mobility compared to the fully compressed monolayers. [125, 137-140] Nevertheless, the effect of template adaptability in determining the crystal orientation was hardly discussed.

In order to elucidate the relationship between template organization and crystal orientation and to get more insight into the template adaptability to the developing mineral phase, systems with tunable properties need to be designed. Self-organized organic templates with adjustable flexibility and in which the distances between the functional groups can be changed in a controlled manner would offer the possibility to address these issues.
1.4 Aim and outline of the thesis

The research described in this thesis applies concepts from Nature to create biomimetic structures, which can be used to study and elucidate processes and interactions in biological systems. More specifically block copolymer films are employed to create patterns that can be used to study the formation and organization of muscle tissue in vitro, whereas mineralized polymer patterns are used to monitor the interaction of bone cells with calcium carbonate (CaCO₃)-based biomaterials. In addition self-organizing supramolecular structures with a defined chemistry and organization are employed to investigate the mechanisms of CaCO₃ crystallization controlled by organic templates.

The first part of the research focuses on using patterned films of amphiphilic ABA block copolymers to control muscle cell attachment and alignment, as well as the patterned deposition of CaCO₃ films. Chapter 2 presents the selective adhesion/delamination behavior of POEGMA-based copolymer films deposited on hydrophobic and hydrophilic substrates, which is the result of the block copolymer self-organization. The selective delamination from hydrophilic regions of a substrate upon exposure to an aqueous solution is exploited in Chapter 3 to generate, in one step, patterned substrates of copolymer-coated gold lanes on glass. The cell-resistant POEGMA groups at the surface of the polymer lanes are used to induce the alignment of C2C12 mouse myoblasts on the uncovered glass lanes. The myoblasts are differentiated into 400-500 µm long, parallel oriented myotubes that show contraction upon electrical stimulation. In Chapter 4, conducting PEDOT substrates are photolithographically patterned using the ABA polysilane-based block copolymers, which do not show the selective delamination. These substrates are successfully employed to control muscle cell attachment and alignment. In addition, their surface properties can be controlled electrochemically, either by deposition of electroactive materials or by switching of the oxidation state. In Chapter 5, the photolithographically patterned films of the ABA polysilane-based block copolymers are used, in combination with the possibility to shape amorphous calcium carbonate in its amorphous state, to create patterns of calcium carbonate films. Preliminary cell culture experiments show that rat bone marrow stromal cells are able to attach and differentiate into osteoblast-like (i.e bone producing) and osteoclast-like (i.e. bone resorbing) cells on the crystalline CaCO₃ films. Therefore, the patterns of CaCO₃ films may serve as a 2D model system for CaCO₃ biomaterials with which the interaction of bone cells with the mineral can be studied.

The second part of the research concentrates on using self-organized systems based on supramolecular interactions as 2D templates for controlled CaCO₃ crystallization. In Chapter 6 and Chapter 7, self-organizing Langmuir monolayer systems are employed to study the relationship between the organization and flexibility of the template and the polymorph, morphology and orientation of CaCO₃ crystals nucleated underneath. The first system, presented in Chapter 6, is based on a water soluble (Leu-Glu)₄ octapeptide modified with a phospholipid tail in order to increase its amphiphilicity. This lipopeptide forms a β-sheet structure at the air-water interface thereby exposing an ordered array of carboxylic acid groups to the aqueous phase, which acts as a biomimetic mineralization template for the formation of a new crystal habit of calcite. In Chapter 7, monolayers of bisurea-based amphiphiles, consisting of an alkyl chain, a bisurea unit and different amino acids as head groups are used. By varying the amino acid head group (i.e. from glycine to alanine to valine), it is possible to control the packing and the flexibility of the monolayer templates. The most
flexible valine-based monolayer acts as the best template, promoting the formation of uniformly oriented calcite crystals. The results of the mineralization experiments performed using the monolayer systems described in Chapter 6 and Chapter 7 demonstrate that the capability of a monolayer template to direct nucleation and growth of uniformly oriented crystals is related to its ability to adapt to the structure of the inorganic phase. Chapter 8 describes the use of films of a bisurea-based thermoplastic elastomer, surface-modified by addition of an amine functionalized bisurea molecule, as 2D templates for controlled CaCO$_3$ crystallization. The additive molecules form well-organized ribbon-like aggregates, exposing an array of NH$_3^+$ functional groups, not integrated in the polymer film structure but persistent at the surface. These aggregates induce the specific nucleation of the vaterite polymorph and a specific crystallographic orientation of the crystals. These surface-modified polymers offer in principle the possibility to fabricate 3D scaffolds consisting of a mesh of polymer fibers coated with an organized array of functional groups. After mineralization, these 3D scaffolds could be used for bone cell culture, with possible applications in bone tissue engineering.
1.5 References and notes

[43] BSA - bovine serum albumin
[44] PLGA - poly(lactic-glycolic acid)
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Y.-J. Han, J. Aizenberg, J. Am. Chem. Soc. 2003, 125, 4032.


POEGMA - poly[oligo(ethylene glycol) methyl ether methacrylate]
Chapter 2

Surface induced selective delamination of amphiphilic ABA block copolymer thin films

Abstract

This chapter describes the unforeseen property of selective adhesion/delamination of amphiphilic block copolymer films in coating hydrophobic and hydrophilic substrates. When spincoated from THF solutions onto hydrophilic substrates (e.g. glass and O$_3$-treated silicon wafer) amphiphilic poly[oligo(ethylene glycol) methyl ether methacrylate] (POEGMA)-based ABA block copolymers $1$ and $2$ form thin films with hydrophobic surfaces. Upon exposure to water these films undergo a fast rearrangement to a hydrophilic surface before they delaminate from the substrate. In contrast, when deposited on a hydrophobic substrate (e.g. Au, Si, Ag), the surface rearrangement of the same copolymer films is less pronounced and they remain as coherent thin films on the substrate. From contact angle measurements it becomes clear that the delamination is accompanied by a rapid surface rearrangement from a hydrophobic to a hydrophilic nature. The extent of surface reorganization of the copolymer films over hydrophobic surfaces is less significant, despite the identical constitutions of the copolymers and identical microphase separated surface morphologies. It is revealed that, within the range of polymers investigated, this behavior is only observed for POEGMA-containing triblock copolymers. Moreover, it is also shown that in order to exhibit this delamination behavior the polar A-block must be large compared to the apolar B-block. Based on XPS and AFM data it is proposed that that this selective adhesion/delamination is a direct consequence of the self-organization of the block copolymers in the polymer thin films.

2.1 Introduction

The ability of copolymers consisting of chemically distinct polymeric segments to undergo microphase separation as a result of enthalpically driven segregation has led to a remarkable range of nanostructured morphologies being catalogued and studied. [1] Consequently, such materials have been the subject of intense study for over ten years. [2-4] Block copolymer thin films show many of the morphologies displayed by the bulk materials, but substrate and surface effects can play a much more pronounced role in the self-organization, particularly for very thin films. A large number of potential applications for these self-organizing thin films have been proposed and demonstrated. Examples include applications as lithographic masks, [5, 6] photonic materials, [7-9] and nanostructured membranes. [10, 11] Amphiphilic block copolymers show the ability to self-organize both in solution and when deposited as films on substrates, thus offering the possibility to modify the interfacial properties. [12] In particular their application in the biomedical field (e.g. as drug delivery systems, [13] biomimetic membranes, [14] surfaces for controlled protein and cell adhesion [15] etc.) has received considerable interest.

Investigation of the self-organization behavior of films of amphiphilic poly[oligo(ethylene glycol) methyl ether methacrylate] (POEGMA)-based ABA block copolymers has lead to the discovery of their unforeseen property of selective adhesion/delamination in coating hydrophobic and hydrophilic substrates, which is described in the present chapter. This selective adhesion/delamination is thought to be a direct consequence of the self-organization of the block copolymers in the thin films. The self-organization is discussed in terms of bulk, surface and substrate energetic interactions, determined by the chemical structure of the copolymers.

The block copolymers of interest in this study were poly[oligo(ethylene glycol) methyl ether methacrylate]-block-styrene-block-oligo(ethylene glycol) methyl ether methacrylate], POEGMA-PS-POEGMA (1) and poly[oligo(ethylene glycol) methyl ether methacrylate)-block-methylphenylsilane-block-oligo(ethylene glycol) methyl ether methacrylate], POEGMA-PMPS-POEGMA (2), which had been synthesized via atom transfer radical polymerization (ATRP) techniques. [16-18]

2.2 Surface-induced selective delamination

It was observed that, when spincoated from THF solutions onto hydrophilic substrates (e.g. glass and O₃-treated silicon wafer) without further treatment (i.e. annealing), amphiphilic POEGMA-based ABA block copolymers 1 and 2 (Scheme 2.1) formed thin films with hydrophobic surfaces. Upon exposure to water these films underwent a fast rearrangement to a hydrophilic surface before they delaminated from the substrate. The delamination resulted in polymer fragments floating in the water as was observed by optical microscopy. In contrast, when deposited on a hydrophobic substrate (e.g. Au, Si, Ag), [19] the surface rearrangement of the same copolymer films was less pronounced and they remained as coherent thin films on the substrate (Figure 2.1).
Figure 2.1 Water contact angles (θ) of different substrates. The block copolymer films (1a-d, 2a-d) selectively delaminate only from the substrates with a hydrophilic surface upon immersion in H₂O.

The thin films of block copolymers 1 and 2 were prepared by spincoating from THF solutions (5 mg/mL) onto the different substrates and were studied as cast. The films of the polysilane derived copolymers 2a-d (POEGMA-P MPS-POEGMA) all exhibited contact angles with water (θ) in the range 85±5°, pointing to a rather hydrophobic film surface. However, for the films spincoated on glass, a decrease of 35±5° was recorded after 10 s of contact with the water droplet, indicating a fast rearrangement of the film surface. [20] In the same time period, the films deposited on hydrophobic substrates showed a maximum decrease of 10°, thus retaining their hydrophobic character.

Scheme 2.1 Structure of the ABA block copolymers used in this study (molecular weight characteristics are given in Table 2.1).
A rapid decrease in the water contact angles was also observed for the polystyrene derived polymers 1a-d (POEGMA-PS-POEGMA) when spincoated on glass, whereas a smaller change was observed when they were deposited on hydrophobic substrates (e.g. Au, Ag, hydrophobic Si wafer). The resulting films surfaces were hydrophobic (θ = 90 ± 10°). [21]

This substrate-induced difference in stability of the polymer films appeared to be dependent on the hydrophilic:hydrophobic weight ratios between the blocks (r), which are given in Table 2.1. The spincoated films of the copolymers with low values of r (i.e. 1e, 1f and 2e) did not show any surface rearrangement or delamination behavior on hydrophobic or on hydrophilic substrates. Films of these polymers all revealed rather hydrophobic surfaces as was deduced from the water contact angles, which were stable and ranging from θ = 80° to 90° (only for 1f: θ = 67±1°).

Table 2.1 Molecular weight characteristics of the ABA block copolymers (structures given in Scheme 2.1).

<table>
<thead>
<tr>
<th>Copolymer</th>
<th>M_n</th>
<th>M_w/M_n</th>
<th>DPA block b*</th>
<th>DPB block a*</th>
<th>r c</th>
</tr>
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<tbody>
<tr>
<td>1a</td>
<td>30 000</td>
<td>1.26</td>
<td>79</td>
<td>86 b</td>
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<td>1b</td>
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<td>56</td>
<td>75 b</td>
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<td>75 b</td>
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<tr>
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<td>16</td>
<td>75 b</td>
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<td>27</td>
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<tr>
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<td>204 b</td>
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</tr>
<tr>
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<td>-</td>
<td>43</td>
<td>0.92 d</td>
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</tbody>
</table>

DP_A block = m; DP_B block = n (for 2-4) and 2n (for 1).

a from SEC measurements (polystyrene standards) unless otherwise noted.
b from 1H NMR spectroscopic analysis in CDCl3.
c r = weight ratio calculated from 1H NMR using formula (2M_n A block/M_n B block) unless otherwise noted.
d weight ratio calculated from SEC using formula (2M_n A block/M_n B block).

Control films were prepared from a series of ABA block copolymers: poly(hydroxyethyl methacrylate-block-methylphenylsilane-block-hydroxyethyl methacrylate), PHEMA-PMPS-PHEMA 3, poly(methyl methacrylate-block-methylphenylsilane-block-methyl methacrylate), PMMA-PMPS-PMMA 4 and poly(methacrylic acid-block-methyl methacrylate-block-methacrylic acid) PMAA-PMMA-PMAA. [22] These films were all stable in aqueous media both when spin-coated on glass and on gold, with stable values for θ of 85±5° (3 and 4)
and of 65±5° (for PMAA-PMMA-PMAA), similar to the case of the “non-peeling” block copolymers 1e-f and 2e. These experiments indicate that the observed phenomena are not common for amphiphilic ABA block copolymers in general, and it is tentatively proposed that the presence of the POEGMA block may be critical. However, a broader range of ABA amphiphilic block copolymers needs to be tested in order to reach a definite conclusion.

2.3 AFM investigation of the copolymer films

In order to further investigate this remarkable behavior, the surfaces of the pristine films of a series of POEGMA-PMPS-POEGMA and POEGMA-PS-POEGMA copolymers were analyzed by Tapping Mode AFM.

![AFM images](image)

**Figure 2.2** Tapping-mode AFM height and phase images of films of (A) 2a, (B) 1e, (C) 2e and (D) 1f copolymers spincoated on glass from THF solution with a concentration of 5 mg/mL. Scan area: 1x1 µm². Vertical scale of height images: 40 nm; phase shift: 40°.
These measurements revealed smooth surfaces ($\sigma_{\text{RMS}} \approx 0.5$ nm for a scan area of 1x1 $\mu$m$^2$) displaying lateral microphase separation for all the polymers exhibiting the selective peeling behavior (Figure 2.2 A, B). Microphase separation has previously been demonstrated for the PMPS derived block copolymers in bulk state by Differential Scanning Calorimetry. [16, 17] Surprisingly, similar morphologies were observed for films spincoated on glass and on gold. To further investigate this, a statistical analysis of the phase images based on the Power Spectral Density was also performed. PSD analysis describes the average surface morphology and it is calculated by integrating the Fourier transform of the topographic profile over the spatial frequency range. The correlation length (determined from the PSD curves) reflects the average size of the domains observed in the phase image. [23, 24] Analysis of the PSD curves extracted from the phase images of films of 2a spincoated on gold and on glass substrates have led to comparable values of the correlation lengths ($\xi = 15$ nm) on both surfaces (Figure 2.3). This indicated that both samples had the same domain size and therefore a similar morphology. The thickness of the films was determined after scratching the film surface (2a, 2e) to be 35±5 nm. It is known that the morphology of thin films of which the thickness is of the same order of magnitude as the phase separated domains (as it is the case also here) can be significantly influenced by the substrate, since the interactions of the different blocks with the substrate can favor specific assemblies. [25, 26]

![Figure 2.3](image)

**Figure 2.3 (A, B)** Tapping-mode AFM phase images of films of 2a spincoated on gold (A) and on glass substrate (B). Phase shift: $40^\circ$. (C, D) Two-dimensional power spectral density (PSD) curves calculated from the phase images. Correlation length ($\xi$) is around 15 nm both for the polymer film deposited on gold and for the film on glass, indicating the same domain size.
AFM imaging of a film of 2a that had been soaked in water for 25 min showed the detachment of the polymer from glass. However, the polymer film was still present in some regions with the nanostructure observed for the pristine films still visible. In contrast, the polymer film on gold appeared unchanged (for details see Chapter 3).

Nevertheless, in the case of the “non-peeling” polymers (1e, 1f, 2e), the films were relatively rough ($\sigma_{\text{RMS}} = 4-5$ nm) and exhibited a granular surface morphology without regular microphase separated structures at the surface of the films (Figure 2.2). These block copolymers have hydrophilic:hydrophobic weight ratios of $\sim 1$ or less and contain a middle hydrophobic block (PS or PMPS) longer than the hydrophilic block (POEGMA). This allows the formation of micelle–like structures, in which the hydrophobic block encapsulating the POEGMA blocks is present at the film surface as a result of the minimization of the surface energy. [27, 28] This might make it more difficult for water to penetrate into the films and to induce delamination.

### 2.4 XPS investigation of the copolymer films

XPS spectra acquired at $0^\circ$ with respect to the surface normal revealed identical chemical compositions for the upper 7-10 nm of the pristine films of 2a both when spincoated on glass and on gold-covered glass (Figure 2.4). The chemical composition of both these film surfaces deviated only slightly from the calculated bulk composition of the polymer film. On the film surface the C1s signal corresponding to the polymer backbone and the phenyl groups (a) was slightly enhanced compared to the C-O (b) and O=C-O (c) peaks, which indicates a slight accumulation of the PMPS block at the surface of the copolymer film. For spectra acquired at 60°, this increase becomes more significant, confirming the enrichment of the unfunctionalized carbon content at the outermost surface of the film ($\sim 20\%$ more than in the bulk of the film). Indeed, it is known that for amphiphilic block copolymers the segments of lower surface energy (e.g. the hydrophobic polymer chains) accumulate at the outermost surface region of the copolymer films under dry conditions. [20, 29]

![Figure 2.4 Carbon peak (C 1s) in the XPS spectra of 2a films spincoated on gold and on glass as spincoated (1) and after immersion in water for 1 min (2). Spectra acquired at $0^\circ$ with respect to the surface normal.](image-url)
After exposure to water of a film spincoated on glass, the relative intensity of the C-O peak (b) increased indicating a clear shift to a higher POEGMA content of the surface top layer (Figure 2.4). Moreover, an additional Si peak (not shown) corresponding to SiO₂ (glass) was visible in the spectrum of the polymer film soaked in water. [30] This is in accordance with the optical microscope observations that showed the appearance of holes in the polymer film on glass. The surface reconstruction of amphiphilic block copolymer films upon exposure to water is a known phenomenon. [20] After wetting, the hydrophilic domains migrate towards the surface in order to reduce the free energy of the water/polymer interface. However, in the present case no change in the XPS spectrum (acquired at 0°) of the polymer film spincoated on gold was observed after exposure to water. This indicates that, although the small change in water contact angle (10° decrease in 10 s) pointed to some degree of surface reorganization, no significant permanent surface rearrangement of the copolymer occurs in the film when gold is used as a substrate.

2.5 Proposed mechanism for the selective delamination process

The most common morphologies encountered for microphase segregated block copolymers include spherical, cylindrical, gyroid and lamellar structures. [1-3] The exact morphology is determined by the relative volume fractions of the distinct polymeric segments. In thin films, as well as the volume symmetry of the copolymer, the symmetry of the surface energy conditions must be taken into consideration. [31-35] All of these morphologies have been observed in block copolymer thin films. [36, 37] However, where strongly asymmetric and antisymmetric boundary conditions [38] exist for block copolymers (as they do for the copolymers presented in this chapter) unusual hybrid morphologies have been predicted and observed. [25, 33, 39, 40] In order to explain both the apparent identical surface morphologies over gold and glass as well as the selective delamination from glass, the following hybrid structures have been postulated for the copolymer films described here (Figure 2.5). At the surface of the film of 2a (Figure 2.2 A and Figure 2.3) the hydrophobic and hydrophilic blocks phase separate into elongated objects that could be either perpendicular lamellae or cylinders lying parallel to the surface. Lamellae perpendicular to the surface are not commonly observed for non-annealed block copolymer films prepared using a procedure that involves a fast solvent evaporation rate (such as spincoating). [41] However, under these conditions, cylinders parallel to the surface have been reported in several cases. [26, 42] Consequently, the most probable morphology of the films of 2a would consist of cylinders parallel to the surface. AFM investigations revealed the presence of a thin layer of one of the blocks at the interface of the copolymer film with air. Light tapping AFM showed a featureless phase image and the microphase-separated structures (Figure 2.2 A) were visible only when using harder tapping conditions. The slight accumulation of the PMPS block (which has a lower surface energy than POEGMA) at the outermost layer of the polymer film is also supported by the angle resolved XPS measurements presented above. It is further proposed that at the gold surface however a parallel lamellar organization occurs with at least one layer of the hydrophobic segment (polysilane or polystyrene) at the substrate. This layer behaves as a hydrophobic seal for the metal and prevents water from reaching the interface and consequently the copolymer film remains attached.
Chapter 2

29

Glass

Golden

PMPS or PS enriched surface

PMPS or PS

POEGMA

Figure 2.5 Postulated models for the self-organization of the delaminating ABA block copolymers over gold and glass substrates (cross section).

At the glass surface POEGMA resides allowing for permeation of water and the subsequent detachment of the copolymer film. The surface reorganization of the films over the glass surface is a result of the reorientation of the OEGMA chains, (initially pointing towards the substrate) to face the surface. The reason for the lack of such reorganization of the POEGMA segments over the gold substrate is at this stage not clear, but may be related to the poor permeability of water through the film and/or physical constraints to swelling/reorganization imposed by the morphology. The proposed organization consequently would lead to constraints with respect to the rearrangement of the film over gold as compared to glass.

At present it is not clear whether the glass transition temperatures of the respective blocks play a role in the reorganization and delamination behavior. The POEGMA block possesses a low glass transition temperature, viz. -31 to -39 °C for the POEGMA-PMPS-POEGMA copolymers\[16\] and -51 °C for the homopolymer.\[43\] In contrast, PMMA (T_g = 105 °C\[44\]) and PHEMA, (T_g = 110 °C\[45\]) have high glass transition temperatures, which implies that the mobility of these chains may be considered highly hindered compared to that of the POEGMA. However the situation is complicated by the fact that the T_g's of the glassy hydrophilic blocks are likely to drop upon exposure to water vapor.\[46\] Irrespective, the lower mobility of the glassy blocks may lead to an inability of the PHEMA- and PMMA- containing copolymers to reorganize which will prevent delamination.

It should be noted however that the discussion is further complicated by the fact that the T_g in the bulk in many cases is different from that observed in thin films (< 80 nm). For example the T_g's of PMMA and PS have been observed to increase above those of bulk polymers at high interfacial energies and decrease for low interfacial energies.\[47\] In the present study, the blocks of the copolymers may have up to 4 separate interfacial energies (e.g. the OEGMA chains may be phase separated from the PMPS/PS, the surface, the substrate and from the PMA backbone) and furthermore the effect of water swelling of the hydrophilic blocks needs to be considered. Consequently, the actual T_g values may be considerably different from those encountered in the bulk. A more detailed study into the T_g behavior in such block copolymer thin films should carried out to address these issues in the context of the delamination phenomena described here.

Preliminary annealing experiments performed on some of the POEGMA-PMPS-POEGMA 2a-d copolymers (165 °C, 2.5 hrs) revealed a significant change in the wetting properties of the polymer films. The surface of the films spincoated on glass had become hydrophobic (θ = 85±10 °), while the films deposited on gold became more hydrophilic (θ = 30-50°, depending on the composition of the copolymer). Moreover, the selective adhesion behavior was lost, as no delamination was observed after annealing. These observations support the proposed model in which the film stability is related to the morphology.
2.6 Conclusion

Polystyrene and polymethylphenylsilane derived ABA amphiphilic block copolymers possessing poly[oligo(ethylene glycol) methyl ether methacrylate] outer segments with suitable hydrophilic : hydrophobic block ratios display selective adhesion to gold, silver and clean (non-oxidized) silicon surfaces. In contrast, delamination is observed for these polymers from hydrophilic surfaces such as glass and oxidized silicon. Delamination is accompanied by a rapid surface rearrangement from a hydrophobic to hydrophilic nature that is less pronounced for the copolymer films on hydrophobic surfaces, despite the identical constitutions of the copolymers and identical microphase separated surface morphologies. This phenomenon offers a unique approach to the selective coating of metal patterned surfaces with copolymers whose functionality and properties may be varied. The application of this property in generating patterned substrates consisting of alternating lanes of glass and copolymer-coated gold patterns and their use in the patterning and alignment of C2C12 mouse muscle cells is described in Chapter 3.
2.7 Experimental Section

Materials. POEGMA-PS-POEGMA, POEGMA-PMPS-POEGMA, PHEMA-PMPS-PHEMA and PMMA-PMPS-
PMMA and samples were provided by Dr. S. J. Holder, Dr. N. A. A. Rossi and C.-T. Yeoh (University of Kent, UK). [16, 17] All solvents were commercial products (Biosolve Ltd., The Netherlands) and were used as received. Ultrapure water (18 MΩ-cm) was generated using a Barnstead EASYpure® LF water purification system.

Film preparation. Glass substrates (microscope cover glasses, diameter = 16 mm, Menzel-Glasser, Germany) were first cleaned by ultrasonic treatment in acetone, rubbing with soap, rinsing in demineralized water, sonication in isopropanol and finally UV/ozone treatment. The substrates were then transferred to a N2 glove box, where first a Cr (3 nm) and then another metal (Au or Ag) layer (20 nm) were deposited by thermal evaporation under vacuum (5x10^-6 mbar, 1 ppm O2 and <1 ppm H2O). The silicon wafers were cleaned by sonication in isopropanol (hydrophobic surface), followed when required by UV ozone treatment (hydrophilic surface). Films of (co)polymers 1-4 were prepared by spincoating from THF solutions (5 mg/mL) on glass (cleaned by ultrasonic treatment in acetone and isopropanol), silicon wafer and on metal-coated glass substrates. Spincoating was carried out using a spincoater from Headway Research Inc. for 50 s at a rotation speed of 1500 rpm. In order to investigate the changes in the copolymer films after exposure to water by XPS and AFM, films of 2a spincoated on glass and on gold substrates were soaked in ultrapure water for 1 min and 25 min, respectively. Water remaining on the sample films was removed by centrifugal force through spinning the sample at 1500 rpm.

Contact angle measurements. Contact angles were measured on a Drop Shape Analysis DSA 10 apparatus from Krüss, using the sessile drop method at room temperature. Contact angle values were recorded continuously during the first 10 s (2 records/s) after placing a water droplet on the film surface. Each set of measurements was repeated at least 3 times on different positions of the same sample. For gold and glass surfaces without any polymer a decrease in contact angles (θ) of 1-2° after 10 s was recorded (controls).

Tapping Mode Atomic Force Microscopy. Tapping Mode AFM measurements were performed using a MultiMode Scanning Probe Microscope (Nanoscope III) from Digital Instruments, Inc. (Santa Barbara, California). The samples were probed using NSG 10 or NSG 11/A “Golden” Silicon cantilevers (NT-MTD, Moscow, Russia), with a force constant of 11.5 N/m.

X-Ray Photoelectron Spectroscopy. XPS measurements were performed in a VG-Scalab 200 spectrometer using an aluminum anode (Al Kα = 1486.6 eV) operating at 510 VA with a background pressure of 2 x 10^-9 mbar. Spectra were acquired at 0° and 60° with respect to the surface normal. The carbon 1s region was fitted in CasaXPS using 3 gaussian/lorentian synthetic peaks representing the hydrocarbon (a), oligoether (b) and carboxyl function (c) in the POEGMA-PMPS-POEGMA 2 block copolymer, respectively (Figure 2.4). All spectra are presented after charge correction and intensity calibration setting the C1s peaks corresponding to hydrocarbon (a) at a binding energy of 285.0 eV and a peak area of 1000. The samples for XPS have been prepared following the procedure described above (see Film Preparation), with the only difference that square glass plates (30x30 mm²) were used as substrates instead of the round glass plates (diameter = 16 mm).
2.8 References and notes

18. It is known that freshly deposited gold is hydrophilic. However, gold surfaces lower their high surface energy by adsorption of hydrophobic organic compounds from air. Therefore, unless cleaned using techniques such as UV/ozone treatment and used readily afterwards, the surface of gold is hydrophobic (see M. E. Schrader, *J. Phys. Chem.* 1970, 74, 2313). For the experiments presented in this chapter gold surfaces with $\theta = 90^\circ\pm 2^\circ$ were used. The values of the contact angles with water were similar for all substrates and were stable in time indicating that there was no significant variation in surface wettability.
20. The films of 1a peeled from freshly evaporated Cu ($\theta = 57\pm 3^\circ$) as well. However, the same polymer films were stable in water when deposited on older Cu substrates that had $\theta = 85\pm 1^\circ$.
21. PMMA-PMMA-PMAA block copolymers with the hydrophilic: hydrophobic weight ratio ($r = 2$) were kindly provided by A.A. Karanam and Dr. L. Klumperman (Eindhoven University of Technology).
29. The possibility that the additional Si peak ascribed to glass (SiO$_2$) is due to polysiloxane fragments that might result from the photodegradation of the PMPS block cannot be completely excluded (as it is difficult to differentiate between the Si peaks corresponding to glass and polysiloxane fragments). However, this is not very likely, since in the case of the copolymer films cast on gold-coated glass substrates this additional peak is not visible. Both samples had been prepared under similar conditions and, prior to the XPS measurements, they had been both kept in the dark (i.e. covered with Al foil), at room temperature and ambient humidity.
37. Asymmetric conditions refer to AB or ABA block copolymer film systems in which the interaction of a B block with the surface (e.g. air or water) is different than the interaction with the substrate, the later being stronger. Antisymmetric conditions are encountered in a system in which one surface (e.g. air)
attracts B segments, while the other surface (e.g. the substrate) attracts A segments, with a differing magnitude of attraction (see reference [33]).

Chapter 3

The patterning and alignment of muscle cells using the selective adhesion of poly[oligo(ethylene glycol) methyl ether methacrylate]-based ABA block copolymers *

Abstract

In Chapter 2, spin-coated films of poly[oligo(ethylene glycol) methyl ether methacrylate] POEGMA-based ABA block copolymers were reported to selectively adhere to hydrophobic and to delaminate from hydrophilic surfaces upon exposure to water. Exploiting this property, patterned substrates consisting of alternating lanes of glass and copolymer-coated gold are prepared by spincoating these polymers on gold patterned glass substrates. The POEGMA groups at the surface of the polymer lanes are used to induce the alignment of C2C12 mouse myoblasts on the uncovered glass lanes. It is observed that, compared to the delamination in cell culture medium, the seeding of the myoblasts directly onto the copolymer film leads to a significant increase in the delamination rate of the polymer film from the glass lanes while the film on the gold lanes remains undisturbed. After the formation of a complete monolayer of cells the myoblasts are differentiated into 400-500 µm long, parallel oriented myotubes that show contraction upon electrical stimulation, thereby forming a promising 2D in vitro model for muscle.

3.1 Introduction

The development of in vitro model systems for the study of cell-cell and cell-substrate interactions is receiving increasing attention, particularly due to the resulting implications for biomaterials research and tissue engineering. In addition, there is a great need for model systems that can be used to unravel the relations between structure, mechanical responses and function of biological materials, as the mechanical loading of cells is related to many aspects of human health and diseases. For the formation of well-organized biological tissues such as nerves and muscles, in vivo there are guidance signals that precisely control the spatial positioning and orientation of the cells. An essential step, for example, in the development of skeletal muscle involves the fusion of oriented mononucleated myoblasts into parallel-aligned multinucleated myotubes that eventually mature into muscle fibers. Patterned substrates consisting of cell repellent and cell adhesive regions have been used for the spatial control of cell attachment in vitro. In addition, the generation of a functional 2D model of muscle that can be used to study the tissue response to mechanical loading should allow not only the localized attachment and proliferation of these cells but also their parallel alignment, which is a crucial requirement for their fusion into myotubes.

In Chapter 2 the unforeseen property of selective adhesion/delamination of amphiphilic block copolymer films in coating hydrophobic and hydrophilic substrates has been reported. When spincoated from THF solutions onto a hydrophilic substrate such as glass, amphiphilic poly[oligo(ethylene glycol) methyl ether methacrylate] (POEGMA)-based ABA block copolymers 1 and 2 form thin films with hydrophobic surfaces. Upon exposure to water these films rearrange such that a hydrophilic surface is exposed before they delaminate from the substrate. In contrast, when deposited on a hydrophobic substrate such as Au, the same copolymer films rearrange to a lesser extent and remain as coherent thin films on the substrate. From these results it can be anticipated that the application of such films on gold patterns deposited on glass substrates, after exposure to aqueous media, should result in patterns of the POEGMA-based ABA block copolymer - coated gold lanes on glass. As polymers, oligomers and copolymers of ethylene oxides are known to exhibit low protein adsorption and cell adhesion such patterns should be suitable to direct the adhesion and proliferation of cells in vitro. Particularly methacrylate-based comb polymers with pendant oligo(ethylene glycol) side chains have proven to be very efficient in long-term resistance to protein adsorption and cell adhesion and were successfully applied in cell patterning.

This chapter presents the generation of patterned substrates consisting of alternating lanes of glass and copolymer-coated gold, by exploiting the selective adhesion/delamination behavior of POEGMA-based ABA block copolymers, and their application in the patterning and alignment of C2C12 mouse muscle cells. The advantage of the system described here is that it allows, in one step the direction of muscle cell attachment and alignment between the gold electrodes that are made cell resistant by the block copolymer film.
3.2 Patterning and alignment of muscle cells

Patterns of gold lanes (thickness 20 nm, widths 100-600 \( \mu m \)) on glass were obtained by evaporation through a suitable mask. These were covered by spincoating from THF solutions of POEGMA-based ABA block copolymers 1 (\( M_n = 12600, M_w/M_n = 1.8 \)) and 2 (\( M_n = 40200, M_w/M_n = 1.3 \)) (Scheme 3.1).

Scheme 3.1 Structure of ABA block copolymers used

The thickness of the resulting optically transparent copolymer films was 35±5 nm, as determined by Tapping Mode AFM after scratching. Within a few hours after immersion in cell culture medium (see Section 3.5) optical microscopy revealed the appearance of cracks and holes in the films covering the glass lanes (Figure 3.1 A) whereas no changes were observed in the films covering the gold (gold layers of 20 nm thickness are optically transparent). Moreover, Tapping Mode AFM revealed after 25 min of exposure to water, small regions where the polymer film had delaminated from the glass (Figure 3.2 A-B). The polymer film still present on glass showed microphase separated nanostructures similar to the ones reported for the pristine films in Chapter 2 (Figure 3.2 C).\[^{15}\] To the contrary, the polymer film on the gold lanes appeared undamaged (Figure 3.2 D).
The patterning and alignment of muscle cells

Figure 3.1 (A, B) Phase contrast optical micrographs illustrating the selective delamination of the POEGMA-derived block copolymer films from glass upon exposure to cell culture medium. Within 4 hrs from the exposure to culture medium, cracks and holes have already appeared in the polymer film on the glass lanes (A) (as indicated by the arrows). After 24 hrs exposure to cell culture medium, the polymer film has completely delaminated from some of the glass lanes (B). (C-F) Optical micrographs of C2C12 mouse myoblasts attached to the glass lanes where the polymer film has delaminated, after 20 hrs at a low cell seeding density (C-D), 24 hrs at a high cell seeding density (E), and 8 days, after fusion into myotubes (F). [25] Arrows point to the edges of pieces of polymer still present on glass; the myoblasts attach in the holes that had already appeared in the polymer film on glass. (D) is an enlargement of a part of (C). The cells are aligned in the direction of the glass lanes. (dark gray – gold lanes; light gray – glass lanes). (G) SEM image of aligned C2C12 myotubes (13 days after seeding on the substrates). The arrow indicates the direction of the glass lanes. Scale bars: 100 µm (unless otherwise indicated)

Although within 24 hrs after immersion in cell culture medium (pH = 7.4, [26] details about the salt concentration in the cell culture medium can be found in Experimental Section), the polymer film had completely delaminated from a large fraction of the glass lanes (Figure 3.1 B), there were still regions where pieces of the polymer film remained present on glass. In order to monitor the delamination, the decrease of the characteristic \(\sigma-\sigma^*\) transition (340 nm)
of the PMPS backbone \(^{27}\) was followed as a function of time in the UV/VIS absorption spectra. After exposing the polymer film to cell culture medium for 3 days the absorption peak at \(\sim 340\) nm had almost disappeared (Figure 3.3 A), confirming the delamination of the polymer film from glass. These measurements also revealed that the delamination process was significantly faster when the film was exposed to cell culture medium compared to exposure to pure water, as upon exposure of a film to \(\)H\(_2\)O\, after 5 days, the intensity of the peak had only decreased to approximately 30% of its original height. This behavior may be related to the interaction of the salts from the cell culture medium with the ethylene glycol chains of the POEGMA block. It is known that the presence of salts influences the solubility in water of PEG and amphiphilic block copolymers containing PEG blocks. \(^{28, 29}\) In general, cations like Na\(^{+}\), K\(^{+}\) and NH\(_4^{+}\) are known to decrease the cloud point and the solubility of PEG. However, divalent cations such as Ca\(^{2+}\) and Mg\(^{2+}\) were found to increase the solubility in water of PEG (co)polymers, probably due to complex formation between the ions and the coordinating ethylene glycol units. \(^{30}\) The cell culture medium contains both mono- and divalent cations (see Experimental Section – Table 3.1). The faster delamination in culture medium suggests that, in the present case, the effect of the divalent cations may be more significant.

![Figure 3.2 Tapping Mode AFM images of copolymer 1 film spincoated on a patterned gold-on-glass substrate after 25 min exposure to H\(_2\)O. (A-C) Images acquired on the glass lanes. The high (bright) domains in the height image (A) related to soft (dark) regions in the phase contrast image (B) correspond to the polymer. The darker (low) areas in the height image (A) matching the hard (brighter) regions in the phase image (B) can be assigned to glass. Inset in (B): phase image of a clean glass substrate for comparison. In the regions where the polymer was present, the nanostructures observed for the pristine films were still visible (C). (D) Image acquired on a gold lane. The polymer film was not damaged and showed the same morphology as the pristine film (see also Chapter 2). \(^{15}\) Scale bars: 500 nm. Vertical scale of height image: 20 nm; phase shift: 40°.](image)
When the polymer films were exposed to cell culture medium in the presence of C2C12 mouse myoblasts (undifferentiated muscle cells), optical microscopy revealed that cell attachment first occurred in the holes that started to appear in the polymer film (Figure 3.1 C-D). Gratifyingly, within 24 hours from the cell seeding, a pattern of myoblasts (50±10 µm long) was visible and the cells could reach 100% confluence on the glass lanes in between the polymer-coated gold lanes (Figure 3.1 E). As the cell culture medium contains heat-inactivated fetal bovine serum it is reasonable to assume that after the detachment of the polymer film the deposition of proteins from the medium onto the glass substrate will constitute the first step in the cell attachment process. Importantly, these experiments show that the POEGMA-based ABA block copolymer films on gold indeed were resistant to cell attachment, although they had been found to show rather large contact angles with water (85±5º), which have been associated with the presence of a significant fraction of the hydrophobic block at the polymer/water interface (Chapter 2).

Figure 3.3 Selective delamination process of films of 1 from glass. (A) UV spectra of films of 1 deposited on glass, as spincoated (a), after 3 days exposure to H2O (b), after 5 days exposure to H2O (c) and after 3 days exposure to C2C12 cell culture medium (d). (B) Cross sectional schematics of the process of selective delamination upon exposure to cell culture medium and cell seeding on the substrates resulting in the formation of a pattern of non-adhesive copolymer-coated gold lanes next to clean glass lanes where the cells can attach. The cartoon does not reflect the real dimensions of the cells. (C) Schematic representation of the geometry of the gold patterns on glass (top view).

In all experiments, the thin copolymer films were used as cast, without being annealed. Therefore, in part as a result of the high glass transition temperatures of PMPS and PS blocks, it is likely that the films of 1 and 2 have non-equilibrium morphologies. X-Ray Photoelectron Spectroscopy studies of films of 1 revealed that, even if there was an enrichment in the hydrophobic PMPS block within the first 10 nm of the film (about 20% more than in the bulk of the film), the POEGMA block was also present at the surface. In air, it is expected to have an enrichment in the more hydrophobic part of the block copolymer at the surface of the film in order to lower the free energy of the air/polymer interface. It is known however that, upon exposure to water, the surface of amphiphilic block copolymers can reorganize to expose the more hydrophilic part to water. Indeed, a small decrease (maximum 10º) of the water contact angle angles of the copolymer films on gold was observed within 10 s (Chapter 2), pointing to a surface reorganization. In order to further investigate this, contact angle
measurements on films of 1 on gold immersed in water were recorded using the captive air bubble method. With this method, contact angle values of 130±5º were obtained for the copolymer films on gold. Control experiments revealed values of the contact angle with an air bubble of 155±5º for glass, 90±5º for gold and 80±5º for PTFE. [34] When measured in air, the contact angles with water were: 85±5º for films of 1 on gold, <10º for glass (complete wetting), 90±2º for gold and 120±2º for PTFE. While in air the contact angle with water of the copolymer film was similar to the one of gold (i.e. hydrophobic), the captive air bubble method showed that, upon immersion in water, the contact angle of the copolymer film became closer to the one of glass, thus indicating a more hydrophilic surface. These results suggest that the surface of the copolymer film reorganizes to some extent upon exposure to water. The increased hydrophilicity could be explained by the migration of the hydrophilic OEG tails towards the outermost surface of the copolymer film.

Control experiments performed on PMPS films showed that the cells could somewhat attach to these films. However, in time the cells remained poorly spread and most of them detached after rinsing the films with phosphate buffer solution. Most probably, the resistance of the copolymer films to cell attachment is due to the presence of the OEG-tails in the hydrophilic block (as expected), but a contribution of the hydrophobic block cannot be completely excluded. Also surprising was the fact that in the presence of a high density of cells, [25] within 24 hrs all the copolymer film from the glass was consistently removed (Figure 3.1 E). This implies that the cells assist in the delamination of the polymer film, although the mechanism behind this process is not yet understood. It is not clear at this stage whether the improved delamination efficiency in the presence of the cells is due to changes in the medium composition or to the mechanical influence of the cells on polymer peeling. With time, an increasing alignment of the myoblasts was observed, which first started on the edge of the lanes at the interface between cell adhesive and non-adhesive regions (Figure 3.1 D). Within 24 hours after seeding they showed a strong orientation along the direction of the glass lane over the whole width of the lane (Figure 3.1 E). Alignment of elongated myoblasts (50±10 µm long) is recognized as a first step towards cell fusion, [7-9] whereas end-to-end organization of myoblasts is also favorable for their end-to-end fusion. Upon changing to a culture medium with lower serum content, which induces cell differentiation, fusion of the myoblasts into 400-500 µm long, highly aligned myotubes was observed (Figure 3.1 F, G). The dimensions of the myotubes force them to orient in the direction of the glass lanes, as they cannot extend onto the non-adhesive polymer coated gold lanes. As expected, the degree of alignment was dependent on the spacing of the gold lanes, [7-9, 35-37] the highest degrees of alignment being obtained for spacings between 100 and 200 µm.

The change in the morphology and the significant increase in length of the cells observed by phase contrast optical microscopy and SEM indicated that the myoblasts had fused into myotubes. However, the fact that the nuclei of the multinucleated myotubes were still present in the center of the cells and not at their periphery suggested that the cells were in an early stage of differentiation. Further differentiation into myofibers should be characterized by migration of the nuclei to the cell periphery and formation of myosin/actin striations. [38] Recent studies have shown however that formation of characteristic cross-striations is very sensitive to substrate flexibility. [7-9]

Since muscle cells generally require several weeks to differentiate into muscle fibers, the stability of the pattern of cells is an important issue. So far, most of the strategies employed for patterning cells on substrates (including the use of self-assembled monolayers of
The patterning and alignment of muscle cells

oligo(ethylene glycol) substituted alkanethiols as cell-resistant surfaces) have proven efficient only for a short period of time (e.g. 1 week), as in time patterns became overgrown with cells. \[7-9, 17\] Recent advances have shown that non-adhesive surfaces such as mannitol groups, \[17\] interpenetrating polymer networks of polyacrylamide and poly(ethylene glycol), \[7-9\] polyelectrolyte multilayer surfaces composed of poly(acrylic acid) and polyacrylamide, \[11\] elastomeric polyurethane films \[12, 13\] as well as oligo(ethylene glycol) functionalized comb polymers \[10\] were able to maintain cell patterns up to 4 weeks. Also with the present system the fidelity of the aligned myotube patterns could be maintained for at least 4 weeks without invasion of the cells onto the non-adhesive copolymer-coated gold lanes. When no copolymer layer was applied, the cells attached equally well both on glass and on gold and no cell alignment was observed.

As it has been shown that myoblasts can use topographical features as guidance cues \textit{in vitro}, \[39-41\] control experiments were performed using patterns of 100 nm high bare gold lanes, as well as patterned copolymer substrates on top of which a thin layer of gold (~ 10 nm) had been sputtered. On these substrates C2C12 cells attached and proliferated equally well, however, without any preferred orientation (Figure 3.4). These experiments indicate that the alignment of the muscle cells on the substrates described in this chapter was due to the presence of regions with different surface chemistries, and thus a different cell adhesion behavior, rather than to the topographical features of the scaffolds.

\[\text{Figure 3.4 Phase contrast optical micrographs of C2C12 mouse muscle cells on control substrates. (A) Myotubes randomly oriented on a pattern of 100 nm high bare gold lanes as substrate (dark lanes – gold; bright lanes – glass). (B) Randomly oriented myoblasts on patterned copolymer substrates on top of which a thin layer of gold (10 nm) was sputtered. Inset: Cross sectional schematics of the patterned substrate used.}\]

\section*{3.3 Electrical stimulation of muscle cells}

It is known that C2C12 cells can be induced to contract by electrical stimulation, which promotes their differentiation into muscle fibers. \[42, 43\] More, in general it has been demonstrated that electric stimulation enhanced proliferation and differentiation of various types of cells. \[44\] Using the conductivity of the gold pattern 5 ms square pulses of 4 V at a frequency of 2 Hz were applied to neighboring gold lanes. Two adjacent gold lanes on the substrates served as the anode and the cathode, respectively. These preliminary experiments showed that indeed the aligned myotubes in between the two gold lanes contracted
synchronously at the imposed frequency. The extent of local cell deformation, expressed as the
decrease (in %) of the length of the contractile region of the myotubes in a contracted state
compared to the length measured in a rest state (in the absence of the electric field), was in the
range of 2-5 % (physiologically relevant local cell deformations). [45] Even if the samples used
for the electrical simulation experiments consisted of a mixture of myoblasts and myotubes,
contraction only occurred in the myotubes and hence could be considered as a further
differentiation step toward functional muscle fibers. [46] This shows that the resulting
monolayer of myotubes may be regarded as a promising model of a muscle fiber that can be
used to investigate the effect of different external stimuli (e.g. mechanical, electrical or
chemical stimuli) on the development and functioning of muscle cells and tissue.

3.4 Conclusion

In summary the results presented in this chapter demonstrate how the selective
adhesion/delamination behavior of the POEGMA - based polymers can be used to generate a
functional 2D \textit{in vitro} model for muscle tissue. The procedure used to obtain this model is
extremely simple, involving only the spincoating of a polymer solution on a patterned
substrate followed by exposure of the substrate to cell culture medium and cell seeding (Figure
3.3 B). Compared to other cell patterning methods previously reported in the literature, that
proved to be also simple and efficient, [10, 11] the present system is particularly suitable for cell
patterning between electrodes. In addition to the preparation of the patterns of electrodes, the
method described here involves only one step, the spincoating of the copolymer film on the
substrate. Afterwards, upon exposure to cell medium and cell seeding, delamination of the
copolymer film from the hydrophilic regions occurs spontaneously.

In Chapter 2 it has been shown that the selective adhesion/delamination behavior of the
block copolymers 1 and 2 was not restricted to gold and glass substrates. [15] In general, upon
exposure to water the block copolymer films delaminate from various hydrophilic substrates
(contact angle with water, $\theta = 10\text{-}50^\circ$), while being stable on hydrophobic substrates ($\theta = 70\text{-}90^\circ$).
In principle therefore this method would allow for the generation of patterns of cells on a
variety of substrates (with different flexibility), provided that they have been pre-patterned
into hydrophilic and hydrophobic regions.
3.5 Experimental section

Materials. See Chapter 2 (Experimental section)

Substrate preparation. Substrates and polymer films were prepared as described in Chapter 2 (Experimental section) with the exception that for the preparation of the gold patterns both the gold and the underlying chromium layer were deposited through a suitable mask.

Tapping Mode Atomic Force Microscopy. See Chapter 2 (Experimental section). Water remaining on the sample films exposed to ultrapure water for 25 min was removed by centrifugal force through spinning the sample at 1500 rpm.

Contact angle measurements. Static contact angles were measured on a Drop Shape Analysis DSA 10 apparatus from Krüss, using the captive bubble method at room temperature. An air bubble was made using a syringe with a U-shaped needle and brought in contact with the surface of the inverted sample completely immersed into ultrapure water (Figure 3.5).

Figure 3.5 Schematic representation of the experimental set up used for the captive air bubble contact angle measurements.

UV measurements. UV spectra were recorded on a Perkin Elmer Lambda 40 UV/VIS spectrometer. The samples were prepared by spincoating the polymer films on glass microscope cover slips (diameter = 16 mm). After removal from H₂O or cell culture medium, the samples were rinsed with water and dried with N₂.

Cell culture. The established C2C12 mouse skeletal myoblast line (ECACC, Porton Down, UK) was used for the study. Prior to cell seeding the substrates have been immersed in EtOH solution 70 % (VWR) to reduce the risk of bacterial or fungal contamination. The substrates were subsequently washed with phosphate buffer saline PBS solution (0.01 M, pH = 7.4, Sigma) to remove residual ethanol. After washing, the samples were air-dried. C2C12 myoblasts were seeded on the patterned substrates in 12-well culture plates. The cells were cultured at 37 °C, in 5 % CO₂ humidified incubator and fed with fresh medium every 2-3 days. Growth medium consisted of Dulbecco’s Modified Eagle’s Medium (DMEM, BioWhittaker) (Table 3.1), supplemented with 15 % (v/v) heat-inactivated fetal bovine serum (Biochrom AG), 1 % (v/v) nonessential amino acid solution (Biochrom), 20 mM HEPES buffer (Biochrom) and 0.5 % (v/v) gentamycine solution (Biochrom). Upon reaching 80-90 % confluence, myoblast differentiation into multinucleated, rod-shaped myotubuli was induced by switching to lower serum content medium (DMEM supplemented with 2 % (v/v) heat-inactivated horse serum, 1 % (v/v) nonessential amino acid solution, 20 mM HEPES buffer and 0.5 % (v/v) gentamycine solution). The cells were visualized using a phase contrast optical microscope (Carl Zeiss, Axiovert 200M).

Table 3.1 Inorganic salt composition of DMEM [26]

<table>
<thead>
<tr>
<th>Inorganic salt</th>
<th>Concentration (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂ anhydrous</td>
<td>200</td>
</tr>
<tr>
<td>KCl</td>
<td>400</td>
</tr>
<tr>
<td>Fe(NO₃)₂·9H₂O</td>
<td>0.10</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>200</td>
</tr>
<tr>
<td>NaCl</td>
<td>6400</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>3700</td>
</tr>
<tr>
<td>NaH₂PO₄·H₂O</td>
<td>125</td>
</tr>
</tbody>
</table>
Scanning Electron Microscopy (SEM). Prior to SEM investigation, the cells were fixed with 2.5 % glutaraldehyde solution (Sigma) in phosphate buffer PBS (0.01 M, pH = 7.4) for 1 hr at room temperature. After fixation, the samples were washed 2 times with PBS and several times with ultrapure water to remove the salts. The specimens were observed with a Philips ESEM XL30 scanning electron microscope at 1.0 kV, using a secondary electron detector.

Electrical stimulation. For the electrical stimulation experiments, electrical pulses were generated using a Labview pulse generator (National Instruments). Cells were stimulated to contract using 5 ms pulses of 4 V at a frequency of 2 Hz. Two adjacent gold lanes on the substrates served as the anode and the cathode (Figure 3.6); the myotubes to be stimulated were attached to glass in between the two gold lanes. During electrical stimulation, short movies showing the contracting cells were recorded using a digital camera (DSC-S75 CyberShot, Sony). Local cell deformation was estimated from freeze frames and was expressed as the decrease (in %) of the length of the contractile region of the myotubes in a contracted state compared to the length measured in a rest state (in the absence of electric field).

Figure 3.6 Schematic representation of the gold pattern used for the electrical stimulation experiments.
It is known that freshly deposited gold is hydrophilic. However, gold surfaces lower their high surface energy by adsorption of hydrophobic organic compounds from air. Therefore, unless cleaned using techniques such as UV/ozone treatment and used readily afterwards, the surface of gold is hydrophobic (see M. E. Schrader, J. Phys. Chem. 1970, 74, 2313). For the experiments presented in this chapter gold surfaces with $\theta = 90\pm2^\circ$ were used. The values of the contact angles with water were similar for all substrates and were stable in time indicating that there was no significant variation in surface wettability.

It has been observed that the cloud points of aqueous solutions of POEGMA-PS-POEGMA copolymers increased in the presence of CaBr$_2$, while they decreased in the presence of NaCl (Geraldine G. Durand, University of Kent – unpublished results).

It is known that proteins from the serum-containing cell culture medium adsorb onto substrates and mediate the adhesion of cells to the substrate. Most mammalian cells must attach to an underlying protein substrates and are stable in time indicating that there was no significant variation in surface wettability.

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[45] During isometric muscle contraction (i.e. when the muscle is activated, but held at constant length), the local cell deformations are in the range of 2-5% (see also H. van Bavel, M. R. Drost, J. D. L. Wielders, J. M. Huyghe, A. Huson, J. D. Janssen, *J. Biomechanics* **1996**, 29, 1069).
Chapter 4

Patterning conducting polymer substrates for muscle cell culture

Abstract

This chapter describes the development of patterned conducting PEDOT substrates, of which the surface properties can be controlled electrochemically and which can be used to direct the attachment and orientation of muscle cells. To this end the UV photosensitivity of the polysilane based ABA block copolymer PHEMA-PMPS-PHEMA is explored. The resulting substrates, consisting of cell-adhesive (PEDOT-PSS) and non-adhesive (PHEMA-PMPS-PHEMA) regions are successfully employed to control muscle cell attachment and alignment. Using these conducting polymer substrates the electrical stimulation of myotubes, which contract at the imposed frequency when electric potential pulses are applied, is also demonstrated. The insulating block copolymer patterns are subsequently used to selectively electropolymerize EDOT only on the conducting PEDOT-PSS lanes. Removal of the block copolymer results in patterns of PEDOT on PEDOT-PSS that are able to direct the cells in the same manner as observed for the block copolymer patterns. However, in this case it is possible to switch the surface properties of the deposited PEDOT lanes from being cell resistant to being cell adhesive by applying an electric potential. In principle, substrates with locally tunable surface properties may find application in the production of engineered tissues consisting of multiple cell lineages derived from the same stem cell type.
4.1 Introduction

In vivo, cells interact with the surrounding extracellular protein matrix and there are specific guidance signals, which direct cell attachment, orientation and differentiation. [1] In vitro, cell growth and differentiation is generally controlled by addition of medium supplements. However, the interactions between cells and their culture substrates also can play a key role in determining the development of cells. [2-8] In addition, electrical stimulation has been used to enhance proliferation and differentiation of various types of cells, such as nerve, [8, 9] muscle [10, 11] and bone cells. [12] Consequently, electroactive polymers represent a class of materials that are potentially very useful substrates for cell culture, as they offer the possibility to manipulate cell growth and differentiation through the application of electrical pulses. [13]

Most of the work on conducting polymeric substrates for cell culture has focused on polypyrrole (PPy), due to its suitability for cell attachment and proliferation, ease of preparation, as well as its biocompatibility in vivo. [2, 8, 9, 14-19] However, it has been shown that in aqueous media PPy [20, 21] exhibited a rapid decrease of conductivity making it less suitable for long-term application in cell culture experiments. In contrast, poly(3,4-ethylenedioxythiophene) (PEDOT) was demonstrated to be very stable under such conditions, making it an excellent candidate for biological applications. [22-24] PEDOT is a low band gap conjugated polymer, with high electrochemical stability in its oxidized state. In addition, PEDOT can be rapidly switched between conducting (doped) and insulating (neutral) states. [25-27] This is of interest as it has been shown that the shape of endothelial cells could be controlled by switching between different oxidation states of PPy. [2] Furthermore, redox switching has been used for the electrically generated release of growth promoters, incorporated as dopants in PPy films. [28] To date however, the interaction of living cells with PEDOT has remained essentially unexplored.

Scheme 4.1 Structure of (A) PHEMA-PMPS-PHEMA block copolymer and of (B) PEDOT-PSS [29]
In Chapter 3 a functional 2D in vitro model for muscle was generated, exploiting the selective adhesion/delamination behavior of amphiphilic ABA block copolymers to create patterned substrates. Combining the patterning of the substrate surface with the ability to induce a specific cellular behavior by manipulation of the surface properties of the substrate (by applying an electric potential, for example) would allow more precise control over cell attachment and differentiation. [30]

The aim of the research presented in this chapter was to develop patterned conducting PEDOT substrates, whose surface properties can be controlled electrochemically and with which the attachment and orientation of muscle cells can be directed. To this end the UV photosensitivity of the polysilane based ABA block copolymer PHEMA-PMPS-PHEMA ($M_n = 10300, M_w/M_n = 1.9$) described in Chapter 2, (Scheme 4.1 A) was explored. Thin films of this polymer spincast on PEDOT-PSS/PET (Scheme 4.1 B) [31] were photolithographically modified and used as the starting point for the development of patterned conducting PEDOT–based cell culture substrates.

4.2 Development of a patterning procedure

The application of photolithography for the patterning of cells is limited due to two reasons: a) in most cases it involves the use of chemicals toxic to cells and b) it generally requires clean-room facilities, which are inaccessible or inconvenient for most biological laboratories. [32, 33] Nevertheless, it has been demonstrated that photolithography could be successfully employed to generate patterned substrates for controlled cell culture, using different photoresponsive materials. [4, 7, 34-42]

Polysilane-derived polymers are characterized by a σ-conjugated Si backbone and a photochemical lability of the Si-Si bonds [43-45] which allow their application as photoresists in fabrication of micropatterns. [44-46] The UV spectrum of polymethylphenylsilane (PMPS) typically shows a σ−σ* transition of the Si backbone at approximately 330-340 nm, depending on the length and the conformation of the polysilane backbone. [47, 48]

Patterned films of PHEMA-PMPS-PHEMA were obtained by spincasting the copolymer from THF solutions onto PEDOT-PSS/PET substrates, followed by UV irradiation of the film through a mask using a UV lamp with a distribution of wavelengths centered around 360 nm. The fragments resulting after irradiation (consisting mainly of the PHEMA, possibly connected to small fragments of the PMPS block) were selectively removed by development in ethanol (Figure 4.1 A). The photodegradation of PHEMA-PMPS-PHEMA was confirmed by the decrease and concomitant shift to lower wavelengths of the characteristic UV absorption band of PMPS around 335 nm (Figure 4.1 B). [44, 45, 49] Patterns with line widths ranging from 60 to 400 µm were produced by choosing a suitable mask. Surface profilometry showed that polymer lanes with heights of ~ 120 nm (UV irradiation for 3 hrs) were obtained when solutions containing 7 mg/mL of PHEMA-PMPS-PHEMA in THF (Figure 4.1 C) were used. [50]
Figure 4.1 (A) Schematic representation of the patterning procedure. (B) Decrease of the UV absorption peak (at ~335 nm) of PHEMA-PMPS-PHEMA copolymer upon exposure to UV light for different time periods, followed by development in EtOH. (C) Surface profile of a pattern of PHEMA-PMPS-PHEMA on gold, obtained after UV-irradiation through a mask with dimensions of 400/200 µm. (D) Phase contrast optical micrograph showing a pattern of C2C12 myoblasts cultured on UV-patterned films of PHEMA-PMPS-PHEMA on PEDOT-PSS/PET.

Culturing C2C12 myoblasts on PEDOT-PSS/PET substrates had revealed that PEDOT-PSS supported cell attachment, proliferation and fusion into myotubes, making it a suitable material for cell culture in vitro. Consequently, on the patterned substrates, due to the cell repelling properties of PHEMA-PMPS-PHEMA, attachment and proliferation of the cells was restricted to the PEDOT-PSS lanes from which the block copolymer had been removed. This resulted in the formation of patterns of myoblasts oriented along the direction of the lanes within 24 hrs. (Figure 4.1 D). Upon changing to a culture medium with lower serum content (after 2-3 days in culture) the myoblasts (50±10 µm long) fused into multinucleated 500-1000 µm long myotubes (as reported also in Chapter 3), highly aligned in the direction of the pattern lanes (Figure 4.2). The fidelity of the patterns was demonstrated by the fact that cell patterns monitored for up to 4 weeks showed no invasion of the cells onto the block copolymer lanes.

Fusion into myotubes represents the first step in the differentiation of skeletal muscle. On the 400 µm and 200 µm wide lanes, the cells showed a maximum deviation of 15° from the direction of the pattern lanes (Figure 4.2 A). The best results, however, were observed on the patterns with lane widths of 100 and 60 µm (Figure 4.2 B). In the later case, patterns of single myotubes were obtained for which the maximum deviation was only 5°, representing the highest degree of muscle cell alignment in vitro reported to date. [38, 53-55] These results were in agreement with previous studies, which have shown that the degree of cell alignment increased with decreasing the width of the regions where the cells attached (as mentioned also in Chapter 3). [37, 38, 41, 53, 54] Myoblast alignment is considered essential for their end-to-end
Indeed the longest myotubes (∼ 1000 µm) were observed on the substrates with PEDOT-PSS line widths of 60 µm (Figure 4.2 B). [56-59]

Figure 4.2 Left: Phase contrast optical micrographs of patterns of C2C12 myotubes (7 days after differentiation) on PEDOT-PSS/PET substrates. Dimensions of the pattern are indicated by a / b µm, where a is the width of the lanes where cells grow (PEDOT-PSS) and b is the width of the cell repelling lanes (PHEMA-PMPS-PHEMA). Right: quantification of the degree of myotube alignment, expressed as the angle of deviation (α) from the direction of the pattern lanes. N (number of myotubes used in statistics): 94 (A) and 46 (B). The features visible in the background of B (out of focus) are cells under the PEDOT-PSS/PET substrate, on the bottom of the culture dish.

In order to demonstrate the viability of this in vitro model system for muscle, the response of the C2C12 cells cultured on PEDOT-PSS substrates to electrical stimulation was tested. In these preliminary experiments the electroactive polymer substrate acted as the anode and a square metal grid suspended in the cell culture medium parallel to the cells was used as the cathode (see Experimental section). The aligned myotubes (6 days post-differentiation) formed on the PEDOT-PSS lanes (100 µm – wide) were induced to contract synchronously at the imposed frequency, by applying 5 ms square pulses of 10 V at a frequency of 1 and 2 Hz. In the contracting myotubes, local cell deformations were observed. The extent of the deformation, expressed as the decrease (in %) of the length of the contractile region of the myotubes in a contracted state compared to the length measured in a rest state (in the absence of the electric field), was in the range of 2-5 % (physiologically relevant local cell deformations). [60]
4.3 Chemical modification of the PEDOT surface

It was anticipated that the insulating PHEMA-PMPS-PHEMA patterns on PEDOT-PSS (Figure 4.3 A - a) could be used to selectively deposit new PEDOT on the conducting PEDOT-PSS lanes of the substrate by electropolymerization. (Figure 4.3 A - c). To this end 3,4-ethylenedioxythiophene (EDOT) was electropolymerized on the patterned substrates using a constant potential (1.3 V) and a fixed monomer concentration (5 mM in acetonitrile) (Figure 4.3 B). Indeed transparent blue PEDOT films were selectively deposited on the PEDOT-PSS lanes from which the block copolymer had been removed. UV-VIS-NIR characterization of the films revealed a strong near-IR and mid-IR absorption attributed to PEDOT in its oxidized state (Figure 4.4 A). [25, 27, 61]

The formation of the new polymer lanes was controlled by monitoring the current as a function of time and terminating the reaction after a predetermined amount of charge (Q = 15 mC/cm²) had passed through the PEDOT-PSS electrode (Figure 4.3 A - inset). The chronoamperometric plot showed the three regions characteristic of the formation of an electroactive polymer: [62, 63] an exponential decay corresponding to the charging of the electrode double layer (a), a regime controlled by the diffusion of the monomer to the electrode surface (b) and a region where the constant current value (c) indicates the progress of polymerization.

After removal of PHEMA-PMPS-PHEMA by UV irradiation and development in ethanol (vide supra), a pattern of PEDOT lanes (~ 400 µm wide) and PEDOT-PSS lanes (~ 200 µm wide) was obtained (Figure 4.4 B). Surface profilometry showed that the height of the electrodeposited polymer lanes was ~ 90 nm. [50] Tapping Mode AFM investigation of the deposited PEDOT films revealed continuous films with a granular morphology characteristic of conducting polymers (Figure 4.4 C, D). [63] The grain size was in the range 50-100 nm and the...
films showed a root mean square roughness of ~ 6 nm for a scan area of 1x1 µm² and ~ 45 nm for a scan area of 100x100 µm², respectively.

Although there was no absolute proof that the PHEMA-PMPs-PHEMA copolymer film had been completely removed from the PEDOT-PSS lanes after UV irradiation and development in EtOH, the procedure was adequate both for the electrodeposition of a continuous film of PEDOT and for obtaining a surface that allowed cell attachment and proliferation.

![Figure 4.4 (A)](image1)
![Figure 4.4 (B)](image2)
![Figure 4.4 (C)](image3)
![Figure 4.4 (D)](image4)

**Figure 4.4** (A) UV-VIS-NIR spectrum of a PEDOT film deposited by potentiostatic polymerization. The pre-set value of the charge passed during polymerization was \( Q = 15 \text{ mC/cm}^2 \). (B) Surface profile of a pattern of alternating PEDOT lanes (deposited by potentiostatic polymerization, \( Q = 15 \text{ mC/cm}^2 \); width = ~ 400 µm, height = ~ 90 nm) and substrate (gold) lanes (~ 200 µm), [50] after removal of PHEMA-PMPs-PHEMA. (C-D) Tapping Mode AFM height (C) and phase (D) images of PEDOT films deposited on PEDOT-PSS. Vertical scale of height image: 50 nm; phase shift: 80º.

Surprisingly, the oxidized PEDOT deposited by potentiostatic polymerization also prevented cell attachment (Figure 4.5). It is known that protein adsorption and cell attachment properties of conducting polymer films are affected by the dopant. [8] As PEDOT films are prepared in the presence of TBAPF₆, the incorporation of PF₆⁻ counter ions in the oxidized polymer films may play a role in the cell repellant behavior. Similarly, it has been shown that chlorine-doped polypyrrole also prevented cell attachment, while PSS-doped PPpy promoted cell attachment and proliferation. [8]
This phenomenon was further investigated by applying a negative voltage (E = -0.5 V, CH₃CN, 30 min) \[64\] (Figure 4.6 A) to the as-prepared doped PEDOT films (Figure 4.6 B - a) until the current had decreased to zero. The reduction of the PEDOT was demonstrated by the appearance of a blue-shifted broad absorption around 600 nm in the UV spectrum, attributed to the π-π* transition and characteristic of the neutral state (Figure 4.6 B - b). \[25, 27, 65\] A second absorption band around 900 nm, known to appear at low doping levels \[61\] was also observed.

The undoping of the electrochemically deposited PEDOT lanes resulted in a change of surface properties from being cell resistant (in the oxidized state) to promoting cell attachment (Figure 4.7). C2C12 muscle cells were now able to attach and proliferate on both the PEDOT-PSS lanes and on the electrochemically deposited PEDOT. The precise mechanism by which altering the oxidation state of the electrochemically deposited PEDOT makes it suitable for cell attachment still needs to be investigated. It has been observed that surface charge density of conducting polymer films affected the protein adsorption and cell-substrate interactions. \[13\] Previous studies on PPy showed that proteins adsorbed more efficiently onto oxidized PPy than onto the neutral polymer. \[2, 66\] Accordingly, if in the present case the surface charge density was the only factor determining the cell adhesive properties of PEDOT, protein adhesion and hence cell attachment should be enhanced on the oxidized polymer. \[67\] However, the opposite effect was observed. This suggests that the concentration of PF₆⁻ ions in
the film (high for the doped polymer and low for the undoped polymer) plays a significant role in determining the cell adhesive properties of PEDOT films.

![Figure 4.7 Phase contrast optical micrographs of C2C12 cells cultured on substrates consisting of PEDOT-PSS (region a) next to PEDOT deposited by potentiostatic polymerization (region b). Oxidized PEDOT (A) prevents cell attachment, while cells can attach and proliferate on PEDOT in its partially reduced state (B).](image)

4.4 Conclusion

PEDOT-PSS substrates were coated with a photolithographically patterned film of PHEMA-PMP-PHEMA copolymer giving rise to cell adhesive and non-adhesive regions. These substrates were successfully employed to control muscle cell attachment and alignment. Using these conducting polymer substrates the electrical stimulation of myotubes, which contracted at the imposed frequency when electric potential pulses were applied, was also demonstrated.

The insulating block copolymer patterns were subsequently used to selectively electropolymerize EDOT only on the conducting PEDOT-PSS lanes. Removal of the block copolymer resulted in patterns of PEDOT on PEDOT-PSS that were able to direct the cells in the same manner as observed for the block copolymer patterns. However, in this case it was possible to alter the surface properties of the deposited PEDOT lanes from being cell resistant to being cell adhesive by applying an electric potential. This approach may find application in generating patterns of different cell populations, which would offer the opportunity to study heterotypic cell-cell interactions. [68] Moreover, substrates with locally tunable surface properties may find application in the production of engineered tissues consisting of multiple cell lineages derived from the same stem cell type. [30]
Patterning conducting polymer substrates for muscle cell culture

4.5 Experimental section

**Materials.** See also Chapter 2 (Experimental section). The PEDOT-PSS/PET substrates (ORGACON foil) were provided by Dr. L. Groenendaal (AGFA - Gevaert N. V., Belgium). EDOT monomer was purchased from Bayer and TBAPF$_6$ (tetrabutylammonium hexafluorophosphate) from Fluka. Tetrahydrofuran (AR), ethanol (AR) and acetonitrile ([H$_2$O] < 10 ppm) were obtained from Biosolve Ltd.

**Photolithographic patterning.** Films of PHEMA-PMPS-PHEMA were prepared by spincoating 7 mg/mL solutions in THF on PEDOT-PSS/PET substrates and gold-coated glass plates [prepared as described in Chapter 2 (Experimental section)]. Spincoating was performed using a CHEMAT Technology spin coater KW-4A at 500 rpm (8 s), followed by 1500 rpm (40 s). The spincoated films were selectively irradiated with UV light (Spectroline® UV lamp, USA, with a distribution of wavelengths centered around 360 nm) through a photomask (3 hrs). After UV irradiation, the samples were developed in ethanol (30 min), resulting in the selective removal of PHEMA-PMPS-PHEMA from the irradiated copolymer regions.

**UV-VIS-NIR spectroscopy.** UV and UV-VIS-NIR spectra were recorded on a Perkin Elmer Lambda 900 UV/VIS/NIR Spectrometer.

**Surface profilometry.** Height profiles of the patterns were measured on gold-coated glass substrates (~ 1 mm thick), using a Tencor P10 profilometer (scan length: 2 mm, scan speed: 0.05 mm/s, tip force: 8 mg).

**Electropolymerization.** All experiments were performed using Ecochemie Autolab PGSTAT T30 and a home-made electrochemical cell. In the experimental setup, the working electrode (PEDOT-PSS or gold-coated glass plates) was placed on a Teflon holder, in electrical contact with the electrolyte solution contained in a glass vessel (~ 2 mL). A platinum disk was used as the counter electrode and Ag/AgCl wire as the reference electrode (Fc/Fc$: +0.40$ V, acetonitrile, TBAPF$_6$). Before electropolymerization started, all solutions were purged by argon.

Potentiostatic polymerization of EDOT was performed using a solution in acetonitrile containing 0.1 M TBAPF$_6$ and the monomer (5 mM). The applied potential was 1.3 V (vs. Ag/AgCl). Polymerization was automatically stopped after a pre-set value of charge (Q) was reached. Current integration for the calculation of Q was carried out using Autolab software (Ecochemie). PEDOT films were prepared using $Q = 15$ mC/cm$^2$. The polymers were deposited as blue films on the working electrode. After polymerization the films were washed with acetonitrile and dried in a stream of N$_2$.

**Cell culture experiments.** See Chapter 3 (Experimental section).

**Electrical stimulation.** The experimental procedure is described in Chapter 3 (Experimental section). The experimental set-up is depicted in Figure 4.8. A home-made metallic grid (sterilized by autoclaving prior to use), placed at ~ 2 mm from the conducting polymer substrate was used as the cathode.

![Figure 4.8](https://via.placeholder.com/150)

**Figure 4.8** Schematic representation of the experimental setup used for electrical stimulation of muscle cells. Dimensions are not to scale.
4.6 References and notes

[31] Commercially available, highly conducting and flexible poly(3,4-ethylenedioxythiophene) - poly(styrenesulfonate) (PEDOT-PSS) coating deposited on a poly(ethylene terephthalate) (PET) film, 125 μm thick (ORGACON foil, AGFA-Gevaert N. V.).
Patterning conducting polymer substrates for muscle cell culture

[50] As no reliable surface profiles could be obtained for the PEDOT-PSS/PET substrates due to their flexibility these measurements were performed on patterns prepared on gold coated glass substrates.
[55] It should be mentioned however that the degree of cell alignment has not been quantified in all the studies that involved control of cell orientation.
[59] In vivo, depending on the species, type of muscle and age of the organism, the length of a myotube ranges from micrometers to millimeters.
[60] During isometric muscle contraction (i.e. when the muscle is activated, but held at constant length), the local cell deformations are in the range of 2-5 % (see also H. van Bavel, M. R. Drost, J. D. L. Wielders, J. M. Huyghe, A. Huson, J. D. Janssen, *J. Biomechanics* 1996, 29, 1069).
[64] Previous investigations showed that thin PEDOT films were dedoped (completely reduced) at -1.0 V (vs Ag/Ag⁺). At an applied voltage of -0.5 V, it is expected to deal with two electronic states, i.e. PEDOT being partially in a neutral state and partially in an oxidized state (see also reference [65]).
Chapter 5

Shaping amorphous calcium carbonate films into 2D models for CaCO₃ biomaterials

Abstract

Patterns of calcium carbonate films with dimensions of 200-400 µm are obtained using the photolithographic properties of the polysilane based ABA block copolymer, PHEMA-PMPS-PHEMA, and the possibility to shape calcium carbonate films in their amorphous state. When the mineral is deposited onto a polymer film previously irradiated with UV-light through a mask, the amorphous CaCO₃ layer grown on the irradiated lanes can be selectively removed upon immersion of the film in ethanol, resulting in the formation of a pattern of mineral film lanes, which upon standing crystallized yielding a mixture of calcite and vaterite. Preliminary cell culture experiments show that rat bone marrow stromal cells are able to attach and differentiate into osteoblast-like (i.e. bone producing) and osteoclast-like (i.e. bone resorbing) cells on the continuous crystalline CaCO₃ films. Therefore, the patterns of CaCO₃ films may serve as a 2D model system for CaCO₃ biomaterials with which the interaction of bone cells with the mineral can be studied.
5.1 Introduction

Bone is a highly organized composite material that has the capacity to heal and remodel itself. During the remodeling process, bone is resorbed by osteoclasts (i.e. bone resorbing cells), which subsequently undergo apoptosis or return to the non-resorbing stage. Their place is taken by osteoblasts (i.e. bone producing cells), which synthesize the new mineralized matrix. Nevertheless, bone self-repair is limited to small bone defects and its ability to regenerate decreases with age and it is affected by diseases. Amongst other materials, biogenic calcium carbonate (e.g. from coral skeletons and sea urchin spines) has been developed as a promising material for bone replacement and regeneration, as it is easily resorbed by osteoclasts and replaced by native bone. Moreover, nacre (present in mollusk shells) shows bone-conductive properties, i.e. it stimulates the activity of osteoblasts, inducing new bone formation. Currently, synthetic, bio-inspired organic-inorganic composites are emerging as new materials for bone regeneration, offering more possibilities to tune biocompatibility, biodegradability and mechanical properties.

In order to monitor the interactions of bone cells with scaffold materials, as well as the bone remodeling process, the development of 2D models would be desirable. A substrate consisting of alternating mineral lanes and non-mineralized lanes can be seen as a cross-section through a 3D porous inorganic scaffold (Figure 5.1) that would allow unlimited transport of nutrients and live observation of cells during their development (e.g. by optical microscopy). In addition, as already mentioned in Chapter 1, 2D patterned substrates consisting of alternating mineralized and non-mineralized regions would also in principle offer the possibility to compare the behavior of the cells on regions with different concentrations of mineral ions (such as Ca\(^{2+}\)). It is known for example that the extracellular calcium concentration controls osteoblast proliferation, differentiation and function and it has been suggested that the gradual dissolution of biomaterials such as hydroxyapatite, releasing Ca\(^{2+}\) ions, might be responsible for their osteoconductive properties.

![Figure 5.1](image)

Figure 5.1 Schematic representation of a 2D model substrate consisting of alternating lanes of mineral (CaCO\(_3\)) and non-mineralized lanes (right), which can be seen as a cross-section through a 3D porous inorganic scaffold such as the piece of coral shown in the left image.

Patterned self-assembled monolayers (SAMs) have been instrumental in obtaining ordered arrays of minerals, patterned calcite single crystals, as well as patterns of continuous mineral films. These patterns generally are produced via the method developed by Aizenberg et al. which is based on diffusion limited crystal nucleation and growth. This approach involves the use of substrates patterned into areas with different nucleating abilities for CaCO\(_3\). It is proposed that in these systems nucleation begins...
at rapidly nucleating sites, enhancing the transport of ions to the growing crystals. Consequently, there is a significant reduction of the concentration of calcium and carbonate ions in the slowly nucleating regions where nucleation is therefore inhibited. However, the application of SAMs is limited to a small number of substrates in principle. Materials and simple methods more generally applicable to the generation of 2D mineral patterns on a variety of substrates are highly desirable.

In Nature many organisms make use of amorphous calcium carbonate (ACC) as a transient intermediate for the production of single crystals of calcite and aragonite with elaborate shapes. Being isotropic, ACC can be deposited in any desired shape and, in addition, can sustain mechanical deformations from all directions. [17, 18] Although in a synthetic environment the ACC is normally unstable and transforms immediately into one of the crystalline forms, it has been shown to play a crucial role as a precursor phase in the preparation of crystalline CaCO₃ films. [19-24]

Thin crystalline films of CaCO₃ can be obtained in the presence of soluble acidic macromolecules, such as poly(acrylic acid) (PAA), poly(aspartic acid) or poly(glutamic acid). [19-34] During the mineralization process the amorphous phase was in several cases temporarily stabilized by these macromolecules. [19-24, 34-36] However, their precise role in this process has not been elucidated yet. Some reports showed that formation of CaCO₃ films required a specific surface such as chitin and its derivatives, poly(vinyl alcohol) or amphiphilic porphyrin monolayers, and it was suggested that the cooperation between the substrate and the acidic macromolecules was crucial for thin film formation. [24, 25, 31, 32] Kato et al. proposed a mechanism of mineral film formation in which the polyelectrolytes played a dual role. On one hand, the polyelectrolyte adsorbed on the substrate binds calcium ions resulting in a high local concentration of ions on the substrate surface, which promotes the precipitation of CaCO₃. On the other hand, the polyelectrolyte acts as an inhibitor of the crystallization in the solution. [25, 30, 31, 33] Other studies revealed that PAA could induce the formation of films of CaCO₃ on a variety of substrates, including glass. [19, 20, 23, 27] Gower et al. proposed a different mechanism for film formation involving a polymer-induced liquid precursor (PILP) process, leading to the deposition of an amorphous precursor film. [20, 22, 23, 36] In a recent study, Cho et al. explained the formation of continuous CaCO₃ films on different substrates by the role of PAA in inhibiting crystallization and aggregation of amorphous calcium carbonate colloids. [19] This reduces the cohesive forces between the ACC particles more than the adhesive force between the ACC and the substrate, resulting in spreading of the ACC on the substrate and the formation of a continuous mineral film. Recently, Cho et al. [34] have reported the formation of amorphous CaCO₃ films even in the absence of additives, provided that short deposition times and high levels of supersaturation were used. In general, due to its high instability and solubility, ACC formed in supersaturated solutions readily transforms into more stable crystalline forms. When a crystal nucleus is formed in an ACC film immersed in a supersaturated solution, the surrounding ACC dissolves and CaCO₃ growth is promoted in the direction normal to the substrate (since there is no additive present in solution to inhibit crystal growth). This leads to the destruction of the ACC film and the formation of large discrete crystals and crystal aggregates. However, if the ACC films are removed early from the mineralization solution, the dissolution-recrystallization process cannot take place and the crystallization occurs only within the film.
The present chapter describes the generation of patterns of calcium carbonate films with dimensions of several hundreds of microns using the photolithographic properties of an amphiphilic ABA block copolymer - poly(hydroxyethyl methacrylate-block-methylphenylsilane-block-hydroxyethyl methacrylate), PHEMA-PMPS-PHEMA (introduced in Chapter 2). As already mentioned in Chapter 4, polysilane-derived polymers show good photolithographic properties due to the photochemical lability of the Si-Si bonds in the σ-conjugated Si backbone and they can be used to fabricate micropatterns. These properties are combined here with the possibility to shape calcium carbonate in its amorphous state.

In a first approach to use these patterns as a 2D model system for CaCO₃ biomaterials the deposition of calcium phosphate by osteoblasts was monitored on films of the separate pattern components. In addition it is shown that the patterns of CaCO₃ films can be used to observe the interaction of osteoclasts with the mineral films using optical microscopy. The pattern dimensions were chosen such as to exceed those of the osteoclasts, which can reach sizes of 50-100 µm.

Scheme 5.1 Structure of the PHEMA-PMPS-PHEMA copolymer

5.2 Calcium carbonate deposition on block copolymer-coated substrates

5.2.1 Amorphous calcium carbonate films

Prior to producing the patterns of calcium carbonate films it was investigated whether it was possible to grow continuous CaCO₃ films on a polysilane-based ABA block copolymer suitable for photopatterning. Cho et al. have shown in previous studies that nucleation and growth of CaCO₃ in the presence of PAA was slow on hydroxy-modified surfaces, but resulted in the formation of continuous uniform films of CaCO₃. Therefore the PHEMA-PMPS-PHEMA copolymer (Mₙ = 6600, M_w/Mₙ = 1.5), consisting of two hydrophilic biocompatible segments (PHEMA) and one apolar polymer block (PMPS) was chosen. In Chapter 2 this polymer was shown to form stable films and in an aqueous environment it may be expected to expose part of its OH groups at the polymer-water interface.

The PHEMA-PMPS-PHEMA was spin cast from THF solution onto glass substrates (thickness of the polymer film ~ 35 nm, as determined by AFM after making a scratch). On this polymer layer continuous films of CaCO₃ were grown by immersing them in an aqueous CaCl₂ solution containing PAA and allowing CO₂ vapor from (NH₄)₂CO₃ to diffuse into these solutions. Surface profilometry showed that the average thickness of the CaCO₃ films was ~ 1 µm. Optical microscopy using crossed-polarizers revealed that the as-prepared films did not display birefringence, except for a few spherical domains (Figure 5.2 A), indicating that the films were amorphous with few embedded crystalline spherulites. The FT-IR spectrum of a fresh CaCO₃ film showed vibrations characteristic for amorphous calcium carbonate at 1489
and 1408 cm\(^{-1}\) (double carbonate vibration), at 1076 cm\(^{-1}\) and two broad bands at 874 cm\(^{-1}\) and 710 cm\(^{-1}\) (Figure 5.2 E). \[^{18, 20}\] Moreover, SEM revealed that the freshly prepared films consisted of spherical particles with diameters smaller than 0.5 \(\mu m\), which are characteristic for ACC (Figure 5.2 B -inset). \[^{19}\] Both SEM and optical microscopy showed that the amorphous films were composed of tablet-like regions, with dimensions in the range of 10-80 \(\mu m\) (Figure 5.2 A, B). \[^{41}\]

**Figure 5.2** (A, C) Optical micrographs under cross-polarized light of (A) a CaCO\(_3\) film (amorphous) within 2 hrs after preparation and (C) a CaCO\(_3\) film (crystalline) 24 hrs after preparation. The films are composed of tablets and the arrows (A) point to edges of the tablet-like regions. (B, D) Scanning electron micrographs of the above films within (B) 2 hrs and (D) 24 hrs after preparation. Inset (B): Higher magnification SEM image. (E) FT-IR spectra of an amorphous CaCO\(_3\) film, within 2 hrs after preparation (1) and of a crystalline CaCO\(_3\) film, 24 hrs after preparation (2). (F) Powder XRD pattern of a crystalline CaCO\(_3\) film.
5.2.2 Crystalline calcium carbonate films

Optical microscopy using crossed-polarizers revealed that, upon standing (i.e. within 24 hrs), the tablet-like regions of the amorphous film transformed into one or more crystalline domains, resulting in a characteristic mosaic structure due to the birefringence of the crystals (Figure 5.2 C). The presence of crystallites (~ 300-400 nm) was also observed by SEM (Figure 5.2 D) and the FT-IR spectrum revealed peaks characteristic for calcite at 1796 cm\(^{-1}\), 1416 cm\(^{-1}\), 876 cm\(^{-1}\) and 713 cm\(^{-1}\) (Figure 5.2 E). The shoulder of the peak around 1416 cm\(^{-1}\) and a very small peak around 1083 cm\(^{-1}\) indicated the presence of some vaterite in the crystalline film. Powder X-Ray Diffraction (PXRD) showed the appearance of Bragg peaks consistent with a film composition of calcite and vaterite (Figure 5.2 F), similar to the results reported by Cho et al. [21, 34] The relative intensities of the XRD peaks were close to those of calcite and vaterite powder reference samples, indicating that the film did not show a preferred crystallographic orientation, as expected for substrates without a pre-organized array of functional groups at the surface. [20]

5.2.3 Calcium carbonate deposition in the absence of poly(acrylic acid)

In order to investigate the role of PAA in the process of mineral film formation, CaCO\(_3\) was deposited on the PHEMA-PMPS-PHEMA films also in the absence of PAA. Under the experimental conditions used, only discrete CaCO\(_3\) crystals were obtained on the copolymer coating when PAA was not present. No continuous mineral film formation was observed. Powder X-Ray Diffraction of the discrete CaCO\(_3\) crystals showed Bragg peaks characteristic of calcite and vaterite (Figure 5.3 A). The orientation of crystals was random, with a slight preference for (001) oriented calcite crystals (20-25 % of the calcite crystals), as evidenced by the increased intensity of the (006) peak compared to the XRD pattern corresponding to a reference sample of randomly oriented calcite powder. [8, 42] SEM investigation revealed that the samples consisted of ~ 60 % calcite crystals and ~ 40 % vaterite crystals (Figure 5.3 B).

5.3 Mineral pattern formation

5.3.1 Patterns of discrete crystals

The formation of mineral patterns was first approached without using PAA as an additive during crystallization. When the copolymer coating was irradiated for 2 hrs using UV light through a mask (with dimensions of 200 \(\mu\)m for the covered lanes and 400 \(\mu\)m for the irradiated lanes), followed by development in ethanol (see also Chapter 4), discrete crystals were obtained both on the non-irradiated and on the developed polymer lanes. In a following step crystals were grown on selectively UV-irradiated copolymer coatings, prior to the development in ethanol. This again led to the formation of similar types of crystals and crystal densities on both the UV-irradiated and the non-irradiated polymer lanes. Subsequent development in ethanol did not result in the removal of the crystals from the irradiated lanes; no change in the distribution of the crystals was observed. However, a 2D pattern of discrete CaCO\(_3\) crystals was achieved after applying an extra step involving peeling of the crystals from the irradiated lanes with adhesive tape (Figure 5.3 C, D). SEM analysis of the crystals
present on the adhesive tape revealed large, flat crystals faces that had been attached to the polymer (Figure 5.3 E).

Figure 5.3 (A) Powder X-Ray diffraction pattern of the discrete CaCO₃ crystals grown on the copolymer film. (B) SEM image of discrete CaCO₃ crystals (calcite and vaterite) on the copolymer film. The crystals are viewed from the side that has been exposed to the mineralization solution. (C) Optical micrograph of a pattern of discrete calcite and vaterite crystals. (D) Schematic representation of the experimental procedure used for the generation of patterns of CaCO₃. Dimensions are not to scale. (E) SEM images of discrete CaCO₃ crystals present on the adhesive tape after peeling. The crystals are viewed from the side that has been attached to the polymer.

The scission of the Si-Si σ-bond in the backbone chain of polysilanes is usually accompanied by the formation of Si-OH or Si-O-Si containing fragments. Therefore, the material resulting after UV irradiation of the PHEMA-PMPS-PHEMA block copolymer should consist mainly of the PHEMA block and small parts of the PMPS block, which should be more soluble in ethanol than the original polymer film. However, the Si-OH containing fragments may react with the glass surface, leading to the partial immobilization of some fragments of the PMPS block on glass. Indeed, AFM and XPS investigation of the patterned substrates revealed that the lanes from where the mineral was removed were still covered with a thin layer of copolymer fragments (Figure 5.4). Tapping Mode AFM showed that both films (i.e. the pristine copolymer films and the film obtained after UV irradiation and development in EtOH) exhibited a granular surface morphology. However, the films had a different grain size and surface roughness (Figure 5.4 A, B). For a scan area of 1x1 µm², the root
mean square roughness was \( \sim 5 \) nm for the pristine copolymer films and \( \sim 3 \) nm for the developed films. The morphology of the pristine films consisted of granules with sizes in the range of 45 to 75 nm, while on the developed polymer regions smaller grains with sizes in the range 20-30 nm, together with some larger grains of 40-45 nm were observed. In the XPS spectra, the relative intensity of the C-O (b) and COO (c) peaks compared to the hydrocarbon peak (a) was lower for the developed copolymer film, indicating a reduced PHEMA content (as expected due to its solubility in EtOH) and an enrichment in PMPS block fragments, which had probably attached to the glass surface. (Figure 5.4 C, D). This was further supported by an increase in the intensity of the Si peak in the XPS spectrum of the developed copolymer film.

5.3.2 Patterns of calcium carbonate films

Without development, the PHEMA-PMPS-PHEMA copolymer coatings UV-irradiated through a mask were also subjected to the mineral film formation process, i.e. using PAA as an additive. Optical microscopy under cross-polarized light revealed the formation of a uniform film of amorphous CaCO\(_3\) on the entire copolymer coating. In this case, however, the amorphous CaCO\(_3\) layer grown on the UV irradiated lanes could be selectively removed upon immersion of the film into ethanol, resulting in the formation of a pattern of continuous CaCO\(_3\) film lanes, without needing the peeling with adhesive tape (Figure 5.3 D and Figure 5.5 A).

[45] An important difference between these patterns of films and the patterns of discrete crystals (Section 5.3.1) was the accuracy of the pattern formation. In the patterns of films, the edges of the lanes were less sharp and in some places small pieces of the amorphous films were still present on the UV-irradiated polymer lanes after development in EtOH. In the case
of the CaCO\textsubscript{3} films, most likely patterning occurs upon exposure to ethanol by the selective detachment of the tablets constituting the amorphous film from the UV-irradiated copolymer regions. The size (10-80 µm) and the shape of the tablets determine the resolution of the patterning procedure. For the patterns of discrete crystals (size of crystals: 15-20 µm) the extra step involving peeling of the crystals with adhesive tape, probably adds to the efficiency of the removal of the crystals from the irradiated copolymer lanes. For the CaCO\textsubscript{3} films, however, this method caused the destruction of the entire film.

As in the case of the non-patterned CaCO\textsubscript{3} films (i.e. continuous mineral films obtained on the pristine copolymer coatings without UV irradiation and EtOH treatment), also the patterns of CaCO\textsubscript{3} films crystallized upon standing, as indicated by optical microscopy using cross-polarizers (Figure 5.5 B). The selective removal of the CaCO\textsubscript{3} however, only occurred when it existed as an amorphous film; after crystallization removal of the film by washing in ethanol proved impossible. This implies that the stabilization of the amorphous phase is crucial for the patterning procedure.

The non-patterned CaCO\textsubscript{3} films were observed to crystallize within 1 hr by optical microscopy. Nevertheless, the mineral films on the patterned substrates stayed amorphous for 2-3 hours under ambient conditions and were only completely crystalline after 24 hrs. This is probably due to the use of ethanol in the patterning procedure, which has recently been found to stabilize ACC.\cite{46} A further stabilization was achieved by increasing the concentration of PAA from 28-65 µg/mL. This extended the lifetime of the amorphous phase to approximately 72 hrs. These higher concentrations of PAA, however, did not further enhance the efficiency of the patterning process.

![Figure 5.5](image)

Figure 5.5 Optical micrograph under cross-polarized light showing a pattern of (A) amorphous CaCO\textsubscript{3} film lanes and (B) crystalline CaCO\textsubscript{3} film lanes.

In general, surfaces containing OH functionalities (such as Langmuir monolayers of amphiphilic molecules containing OH head groups\cite{47,48} and SAMs of OH-terminated alkanethiols\cite{49}) are not considered to be very efficient nucleation templates. They usually lead to the formation of \{10.4\} oriented crystals.\cite{8} However, in the present case, the selective peeling with adhesive tape of the CaCO\textsubscript{3} crystals only from the irradiated polymer lanes implies a strong attachment of the crystals to the pristine PHEMA-PMPS-PHEMA films. Also, XRD data and SEM analysis of the crystals suggest that many crystals have not nucleated from \{10.4\} faces. The slight preference for \{00.1\} oriented crystals implies that the polymer may play an active role in crystal nucleation. Nevertheless, for the crystalline CaCO\textsubscript{3} films no
preferred orientation was observed. This suggests that there is a difference between the formation of discrete crystals, which seem to have nucleated directly from the polymer film and the crystalline mineral film, which has evolved from an amorphous film. Furthermore, while ACC films can be removed from the irradiated copolymer lanes by development in EtOH, the crystalline CaCO₃ films and the discrete crystals cannot. This may imply that there is also a difference in the adhesion of the crystalline and amorphous materials, the former being more strongly attached to the copolymer film. It may be speculated that the crystalline material acts as a "cross-linker" between the PHEMA-rich copolymer fragments (formed by UV irradiation), thus hindering their dissolution in EtOH and removal of the crystals from the irradiated regions. Nevertheless, the nature of the interaction between the copolymer film and the mineral remains at this stage unknown.

5.4 Application of the CaCO₃ films as cell culture substrates

Preliminary cell culture experiments were performed to test whether these patterns were suited to study bone cell-substrate interactions. Rat bone marrow stromal cells (BMSC) were cultured and induced to develop into osteoblast-like (i.e. mineral producing) cells with the aim of monitoring the deposition of new biogenic mineral. These experiments were performed separately on continuous crystalline CaCO₃ films and on films of the polymer coating after irradiation and development, to facilitate the quantitative determination of the alkaline phosphatase (ALP-ase) activity – a marker of early cell differentiation – at different time points. In order to exclude a possible effect of the transition from amorphous to crystalline calcium carbonate on the behavior of the cells, all films were allowed to fully crystallize prior to cell seeding. Tissue culture polystyrene (TCP) was used as a positive control to test whether the cells developed normally in the experimental conditions used.

Phase contrast microscopy showed that the cells attached and spread well on crystalline CaCO₃ (comparable to TCP), while on the irradiated and developed regions the cells were less spread and had the tendency to cluster (Figure 5.6 A, B). The ALP-ase activity of the cells cultured on the CaCO₃ films reached a peak at an earlier point in time than the activity of the cells cultured on the developed polymer films (Figure 5.6 F). This suggests that the cells on the CaCO₃ substrates showed osteogenic differentiation at an earlier point in time than on the polymer substrates. Importantly, on the CaCO₃ films optical microscopy and SEM, in combination with EDX (Energy Dispersive X-Ray) and FT-IR analysis demonstrated the extensive and homogeneous mineral deposition in the form of characteristic small round bulbs of calcium phosphate (Figure 5.6 C) in between fibrous material (Figure 5.6 D). Moreover, the characteristic phosphate (at 1017 and 960 cm⁻¹) and carbonate (874 cm⁻¹) peaks in the FT-IR spectrum of the bulbs revealed that the mineral formed was carbonated apatite. In contrast, on the UV-irradiated polymer film, only small amounts of mineral was formed, present in the form of scattered clusters (Figure 5.6 E).

In order to explore the possibility to monitor also the degradation of the calcium carbonate films due to bone cell activity, BMSC were cultured on the patterned CaCO₃ substrates in the presence of 1α,25-dihydroxyvitamin D₃, which is known to induce cell differentiation into osteoclast-like (i.e. mineral resorbing) cells. It has been previously shown that osteoclast activity is characterized by an alternation of two phases: mineral resorption and migration of the cell along the mineral surface.
Figure 5.6 (A-B) Phase contrast optical micrographs showing the attachment of rat bone marrow stromal cells (day 5) on a CaCO₃ film (A) and a developed PHEMA-PMPS-PHEMA polymer film (B). The cells attached and spread well on CaCO₃ films, except for a few holes in the CaCO₃ films (in which PHEMA-PMPS-PHEMA is exposed) where cells do not attach (indicated by the arrows). (C-E) ESEM images showing new mineral deposition by the osteoblast-like cells after 15 days of culture on CaCO₃ film (C-D) and on the UV-irradiated polymer film (E). In (D) round bulbs of calcium phosphate (deposited by the cells), together with collagen fibers can be observed. [53] (F) ALP-ase activity of the osteoblast-like cells. The ALP-ase activity was defined as the amount of p-nitrophenol (pNp) formed per hour and was normalized to the total DNA concentration. [58] (G) Spectral output from the EDX analysis of the mineral bulbs shown in (C-D). (H) FT-IR spectrum of the mineral produced by the osteoblast-like cells cultured on CaCO₃ films.

Optical microscopy and SEM investigations revealed the presence of large multinucleated cells on the CaCO₃ films. Large multinucleated cells with a similar appearance were identified both on the CaCO₃ films and on control TCPS. On TCPS, these cells were positively stained for the expression of tartrate resistant acid phosphatase (TRAP), which is a marker of the development of osteoclastic properties. [56] The TRAP staining could however not be applied to the cells on CaCO₃ films, since the mineral films were completely damaged during the staining procedure. On the CaCO₃ films, some of these large multinucleated cells were found in lacunae with roughened edges on the otherwise smooth mineral film (Figure 5.7).
These were tentatively interpreted as resorption pits in which osteoclast-like cells had degraded the mineral film. Moreover the system allowed monitoring the migration of one of these cells over the calcium carbonate film using optical microscopy over a period of 33 days (Figure 5.7).

![Figure 5.7 Phase contrast optical micrographs illustrating a possible resorption pit on the CaCO₃ film (encircled region) and an osteoclast-like cell during the migration phase (A) - day 27, (B) - day 29, (C) - day 30 and (D) - day 33 in culture medium.](image)

### 5.5 Conclusion

A method was developed for the generation of patterns of calcium carbonate with dimensions of several hundreds of microns. This was achieved by the deposition of amorphous CaCO₃ films on photolithographically patterned **PHEMA-PMPS-PHEMA** coatings, followed by the selective removal of the amorphous mineral layer from the UV-irradiated copolymer lanes by development in EtOH. Upon standing, these amorphous CaCO₃ patterns crystallized leading to the formation of a mixture of calcite and vaterite.

Cell culture experiments performed on films of the separate pattern components using osteoblast-like cells indicated that calcium phosphate deposition was more pronounced on CaCO₃ films than on the regions without mineral (i.e. the developed **PHEMA-PMPS-PHEMA** films). Furthermore, the patterns of mineral films were used as cell culture substrates to observe the interaction of osteoclast-like cells with the crystalline CaCO₃ films. The ability to monitor cells in situ with optical microscopy suggest that the patterns of continuous CaCO₃ films can be regarded as promising 2D model substrates for bone cells cultures. In addition, the patterning method presented here is not restricted only to glass substrates. In principle, this method would allow for the generation of patterns of CaCO₃ on a variety of substrates, including e.g. conducting polymers (see also Chapter 4). This would offer the possibility to fabricate bone biocompatible devices for the enhancement of bone fracture healing by electrical stimulation. [59]
5.6 Experimental section

Materials. See also Chapter 2 (Experimental Section). Poly(acrylic acid) (PAA, M<sub>w</sub>=2000 g/mol) was obtained from Aldrich, calcium chloride (p.a.) from Merck and ammonium carbonate (p.a.) from Acros Organics. Ultrapure water (18 MΩ⋅cm) was generated using a Barnstead EASYpure® LF water purification system.

Substrate preparation. Films of PHEMA-PMPS-PHEMA copolymer on glass were prepared as described in Chapter 2 (Experimental Section).

Crystallization and pattern generation. CaCO<sub>3</sub> was allowed to crystallize on PHEMA-PMPS-PHEMA films spincoated on glass. Each substrate was supported upside down in 5 mL CaCl<sub>2</sub> solution (1M), to ensure that only crystals nucleated on the copolymer film would be bound to the surface. The whole system was subsequently placed in a closed dessicator containing solid (NH<sub>4</sub>)<sub>2</sub>CO<sub>3</sub> at the bottom. Crystallization of the CaCO<sub>3</sub> resulted from diffusion of CO<sub>2</sub> vapor from (NH<sub>4</sub>)<sub>2</sub>CO<sub>3</sub> into the CaCl<sub>2</sub> solution, according to reactions (1)-(3). [8, 39]

\[
\begin{align*}
(NH_4)_2CO_3(s) & \rightarrow 2NH_3(g) + CO_2(g) + H_2O \\
CO_2 + Ca^{2+} + H_2O & \rightarrow CaCO_3(s) + 2H^+ \\
2NH_3 + 2H^+ & \rightarrow 2NH_4^+
\end{align*}
\]

All experiments were carried out at room temperature for 3 hrs 30 min. This resulted in the deposition of discrete CaCO<sub>3</sub> crystals over the entire copolymer film. When PAA was added to the CaCl<sub>2</sub> solution (28 µg/mL), continuous thin films of CaCO<sub>3</sub> were deposited. The samples were rinsed with ultrapure water and blown dry with N<sub>2</sub>. For mineral pattern generation, CaCO<sub>3</sub> was crystallized on PHEMA-PMPS-PHEMA films previously irradiated with UV light (Spectroline® UV lamp, USA, with a distribution of wavelengths centered around 360 nm) through a photomask (2 hrs). After mineralization, the samples were immersed in EtOH for 40 min to selectively remove the polymer fragments from the UV irradiated regions together with the CaCO<sub>3</sub> grown on top. Subsequently the samples were blown dry with N<sub>2</sub>. When discrete CaCO<sub>3</sub> crystals were grown on the substrates, patterns were obtained only after selective removal of the crystals growing on the irradiated polymer lanes with adhesive tape. In order to extend the lifetime of the amorphous phase, the continuous ACC films used for FT-IR and SEM investigation were also immersed in EtOH (40 min) after removal from the mineralization solution.

Optical microscopy. CaCO<sub>3</sub> samples were examined using a Jenaval polarization microscope with crossed polarizers.

Infrared spectroscopy (FT-IR). Pieces of the CaCO<sub>3</sub> films were collected from the polymer/glass substrates and lightly crushed using a diamond crystal. The spectra were recorded in transmission mode on a BioRad FTS6000 FTIR spectrometer coupled to a BioRad UMA500 Infrared microscope (equipped with an MCT detector), at 4 cm<sup>-1</sup> resolution and co-adding 100 scans. The samples containing the calcium phosphate bulbs deposited by cells were measured using a slide-on ATR accessory on the microscope, with a germanium ATR crystal.

Scanning Electron Microscopy (SEM). The samples were mounted on aluminum stubs with double-sided carbon tape. The specimens were observed with a Philips XL30 scanning electron microscope. For CaCO<sub>3</sub> samples, a secondary electron detector was used at an accelerating voltage of 1.0-2.0 kV.

Prior to Environmental SEM (ESEM) investigation, the samples containing cells were fixed in 4 % (v/v) formalin solution (Merck) in phosphate buffer saline solution (PBS, 0.01 M, pH = 7.4, Sigma) (10 min incubation at 37 ºC). After fixation, the samples were washed 2 times with PBS and several times with ultrapure water to remove the salts. The cells were observed using a GSE detector at 5-7.5 kV.

Tapping Mode Atomic Force Microscopy (Tapping Mode AFM). See Chapter 2 (Experimental Section)

X-Ray Photoelectron Spectroscopy (XPS). See Chapter 2 (Experimental Section). The carbon 1s region was fitted in CasaXPS using 3 Gaussian/Lorentian synthetic peaks representing the hydrocarbon (a), hydroxymethylene CH<sub>2</sub>-OH (b) and carboxyl function (c) in the PHEMA-PMPS-PHEMA block copolymer, respectively (Figure 5.4 C, D).
Shaping amorphous calcium carbonate films into 2D model substrates for CaCO$_3$ biomaterials

**Powder X-ray diffraction (XRD).** XRD data were collected on a Rigaku D/Max-B diffractometer using CuK$_\alpha$ radiation. The XRD pattern was recorded in the range 15º < 2θ < 70º using step scan mode (step size 0.02º, counting time per step 10 s).

**Cell culture experiments.** Rat bone marrow stromal cells (BMSC) were isolated from Wistar rats by the method of Maniatisopoulos. [59] Prior to cell seeding the substrates were immersed in EtOH solution 70 % (VWR) to reduce the risk of bacterial or fungal contamination. The cells were visualized using a phase contrast optical microscope (Carl Zeiss, Axiovert 200M).

**Formation of osteoblast-like cells.** BMSC were isolated from the bone marrow of 6-7 week old male Wistar rats. After isolation, the cells were cultured in culture medium composed of α-MEM (Gibco) with 15 % (v/v) heat inactivated (30 minutes at 56 ºC) fetal bovine serum (Biochrom), 1% (v/v) penicillin/streptomycin (10 mg/mL, Biochrom), 0.5% (v/v) gentamycin (10 mg/mL, Biochrom), 0.5% (v/v) L-glutamine (200 mM, Sigma), 10$^{-8}$ M dexamethasone (Sigma), 10 mM β-glycerophosphate disodium salt (Sigma) and 5 mg/mL L-ascorbic acid 2-phosphate (Sigma).

After one week in primary culture the cells were trypsinized and seeded on the substrates in a density of 20000 cells/cm$^2$. Three types of substrates were used: tissue culture polystyrene (control), CaCO$_3$ film (deposited on PHHEMA-PMP5-PHEMA polymer-coated round glass slides, diameter = 16 mm) and UV-irradiated (2 hrs) polymer film developed in EtOH (supported on round glass slides, diameter = 16 mm). The cells were cultured at 37 ºC, in 5 % CO$_2$ humidified incubator and fed with fresh medium every 2-3 days. The cell samples for the DNA and ALP-ase were prepared on days 5, 8 and 14 as follows: the medium was removed from the wells, the cells on the substrates were washed with phosphate buffer saline, PBS solution (0.01 M, pH = 7.4, Sigma) and scraped off into 1 mL ultrapure water. The cells were then lysed in three freeze-thaw cycles (-80 ºC to 20 ºC).

**DNA assay.** The Hoechst 33258 assay for fluorometric quantitation of DNA was used for the determination of the total amount of DNA in the cell samples. [60] The Hoechst reagent was prepared by adding 25 µl Hoechst stock solution (1 mg/mL) to 10 mL TNE (10 mM Tris, 2 M NaCl, 1 mM EDTA in ultrapure water, pH = 7). A DNA stock solution containing double-stranded calf thymus DNA dissolved in ultrapure water was used to obtain calibration line samples ranging from 0 to 10 µg/mL. For each measurement 100 µL of the Hoechst reagent was added to 100 µL calibration or cell sample in a black 96 well plate (Costar). After 10 min incubation at 37 ºC, the fluorescence was measured at 490 nm using a FL600 fluorescence microplate reader (excitation at 360 nm). All samples were measured in triplicate.

**Alkaline phosphatase activity (ALP-ase).** The ALP-ase activity in the cell samples was determined according to the procedure provided with the alkaline phosphatase kit (Sigma) and described by De Ruijter et al. [61] The procedure is based on the hydrolysis of p-nitrophenolphosphate (pNpp) to p-nitrophenol (pNp) and inorganic phosphate by the ALP-ase. 80 µL of the cell sample, 20 µL of the alkaline buffer solution (1.5 M 2-amino-2-methyl-1-propanol) and 100 µL of 5 mM pNpp solution were mixed together and incubated at 37 ºC for 15 minutes. To stop the reaction, 200 µL NaOH solution (0.5 M) was added. The absorption of the pNp at 405 nm in the resulting solution was measured in triplicate in a 96 well plate (Nunclon) by using the plate reader. The alkaline phosphatase activity was defined as the amount of pNp formed per hour and was normalized to the total DNA concentration.

**Formation of osteoclast-like cells.** The BMSC used in these experiments were isolated from the bone marrow of a 14-week-old female Wistar rat. The cells were seeded directly onto the substrates at a density of 20000 cells/cm$^2$, without primary culture. The culture medium was composed of α-MEM with 15 % (v/v) heat inactivated bovine serum, 1% (v/v) penicillin/streptomycin (10 mg/mL), 0.5% (v/v) gentamycin, 0.05 mg/mL L-ascorbic acid 2-phosphate, 10 mM β-glycerophosphate disodium salt and 10$^{-8}$ M 1α,25-dihydroxyvitamin D$_3$ (Sigma).

**Tartrate resistant acid phosphatase (TRAP) staining.** TRAP staining was performed using a TRAP staining kit from Sigma. The cell culture was fixed (30 s; fixative solution: 4% (v/v) citrate concentrate, 36 % (v/v) ultrapure water and 60 % (v/v) acetone) and stained for the osteoclast-associated enzyme TRAP (1 h at 37 ºC; staining solution: 88 % (v/v) ultrapure water, 4 % (v/v) acetate solution, 4 % (v/v) naphthol ASBII phosphoric acid, 4 % (v/v) tartrate solution and 15 mg Fast Garnet GBC salt). The cells were air-dried and investigated by phase contrast optical microscopy. The multinucleated cells expressing TRAP were stained red.
5.7 References and notes

[41] It is also possible that the tablet-like regions are separated by cracks that develop in the mineral film upon drying.
[45] When mineralization was performed on copolymer films that were selectively irradiated with UV light and developed in EtOH, amorphous CaCO₃ deposition was observed both on the intact polymer lanes and on the developed lanes.
As shown in Chapter 4, cells do not attach at all on the pristine PHEMA-PMPS-PHEMA polymer film. This is the consequence of the PHEMA block, which is known for its properties to resist protein adsorption and cell attachment (see G. Peluso, O. Petillo, J. M. Anderson, L. Ambrosio, L. Nicolais, M. A. B. Melone, F. O. Eschbach, S. J. Huang, *J. Biomed. Mater. Res.* 1997, 34, 327). However, in the case of the UV-irradiated polymer film, after removal of the mineral layer by EtOH, there was less PHEMA present in the film. This may account for the fact that cell attachment on the developed copolymer film was allowed to some extent.


The observation of the vibrations at 1400-1650 cm⁻¹ in the FT-IR spectra is consistent with the presence of collagen (see also reference [55] and S. J. Gadaleta, N. P. Camacho, R. Mendelsohn, A. L. Boskey, *Calcif. Tissue Int.* 1996, 58, 17).


In time, cell proliferation (expressed as the amount of DNA extracted from the cells) decreased on both substrates, associated with the beginning of cell differentiation (see also M. C. Siebers, X. F. Walboomers, S. C. G. Leeuwenburgh, J. G. Wolke, J. A. Jansen, *Biomaterials* 2004, 25, 2019). This is due to the fact that, during the deposition of new mineral, the cells become entrapped within the mineral, which hampers DNA extraction and thus accounts for a decrease of DNA content in time.


Chapter 6

Self-organizing β-sheet lipopeptide monolayers as template for the crystallization of CaCO₃ *

Abstract

The results presented in this chapter demonstrate that the amphiphilic lipopeptide 2, forming stable monolayers with an anti-parallel β-sheet conformation, can be employed as a biomimetic mineralization template for the formation of a new crystal habit of calcite. The effect of lipopeptide 2 and N-acetylated octapeptide 3 monolayers on the crystallization of calcium carbonate is investigated. Also, the way the two monolayers interact with the developing mineral phase is compared. The present system demonstrates that the nucleation of different crystal faces can be achieved depending on the ability of the template to adapt to the structure of the inorganic phase. Furthermore, the results described here indicate that stretching of the template in only one direction allows the reorientation of the template’s functional groups such that the stabilization of different crystal planes can be achieved without the need for an epitaxial relation between the two components.

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6.1 Introduction

The large variation in inorganic structures encountered in biological systems, often with exquisite and unique morphologies, has been fascinating and inspiring researchers in all scientific disciplines for ages. [1] For materials scientists the understanding of the principles underlying the process of biomineralization holds great promise for the design and synthesis of new inorganic and hybrid structures with yet unrealized properties. The central concept in biomineralization research is that organized biomacromolecules, containing well-defined arrays of functional groups, control polymorph selection and oriented nucleation of crystals through lowering the nucleation energy of specific crystal faces. [2] Control over crystal morphology is then exerted by the interaction of biomolecules in solution with specific crystal planes during growth.

Recently it has become clear that the classical concept which proposes that the template, containing well-defined arrays of functional groups, controls the nucleation of the inorganic crystals via geometric and stereochemical matching, needs expanding. [3] Furthermore, it has been shown for several systems, in vitro [4-8] as well as in vivo, [9, 10] that amorphous calcium carbonate (ACC) can play a crucial role as a transient intermediate. The subsequent transformation of the temporarily stabilized ACC into one of the crystalline forms is thought to be controlled by the (bio)macromolecules present in the system. In the last years, it was demonstrated that specific nucleation of calcite could be achieved using self-assembled monolayers, [11, 12] self-organized Langmuir films [13, 14] and helical polymers, [15] without an epitaxial match between the pre-positioned functional groups and the nucleating crystal planes. Also more evidence has become available suggesting that electrostatic interactions play an important role in the control of crystal growth. [16-21]

In Nature, crystal nucleation and growth of CaCO$_3$ is often controlled by carboxylate-rich polypeptides (e.g. aspartate and glutamate) present within a macromolecular matrix with organized $\beta$-sheet domains. [22] Although (poly)peptides have been successfully employed as additives able to modify the growth of calcium carbonate crystals, [23-25] there have been few reports in which peptides with predefined secondary structures have been studied as templates for mineralization. [20, 26-32] Polypeptides composed of alternating Glu and Leu residues have a random coil structure in water, but can adopt a $\beta$-sheet conformation when interacting with a mineral surface. [31] Amphiphilic peptides of different lengths, consisting of alternating hydrophilic and hydrophobic amino acid residues, have also been studied for their influence on the mineralization of CaCO$_3$ both in solution [(Asp-Phe) based peptides], [20] and deposited onto an insoluble substrate [poly(Asp-Leu) and poly(Glu-Leu)]. [32] Recently it was demonstrated that similar amphiphilic peptides comprising phenylalanine (Phe) and glutamic acid (Glu) residues can form ordered Langmuir monolayers with a $\beta$-pleated sheet structure at the air-water interface. [33]

In this chapter, the water soluble (Leu-Glu)$_4$ octapeptide motif 1 modified with a phospholipid moiety (dioleoyl phosphatidyl ethanolamine, DOPE) in order to increase its amphiphilicity is used. The resulting lipopeptide 2 (Scheme 6.1) forms stable monolayers with a $\beta$-sheet organization at the air–water interface, thereby exposing an ordered array of carboxylic acid groups to the aqueous phase. The present chapter describes how this monolayer interacts with Ca$^{2+}$ ions and subsequently is employed as a biomimetic mineralization template for the formation of a new crystal habit of calcite.
Scheme 6.1 Structures of the molecules used in this study

\[
\begin{align*}
1 & \quad R = H \\
3 & \quad R = \text{C}(\text{O})\text{CH}_3
\end{align*}
\]

6.2 β-Sheet lipopeptide monolayers

Surface pressure-surface area (Π-Mma) isotherms (Mma – mean molecular area) of the lipopeptide 2 were characterized by an apparent condensed liquid phase present over a rather large trajectory (Figure 6.1 A). The slope of the isotherm indicated a fluid-like character of the monolayer, most probably provided by the unsaturated lipid chains. Brewster angle microscopy (BAM) images revealed that 2 self-assembled into preformed domains with dimensions in the millimeter range. These domains, which were present already in the expanded state, upon compression fused to form a continuous film (Figure 6.2). CD and FT-IR investigation of compressed monolayers (at Π = 30 mN/m) transferred to quartz plates and ZnSe prisms, respectively, revealed a β-sheet organization (Figure 6.3). The β-sheet structure was confirmed by the appearance of a CD signal displaying a maximum at 202 nm and a minimum at 218 nm, as well as by an amide I vibration at 1628 cm\(^{-1}\) in the FT-IR spectrum. The small IR band at 1694 cm\(^{-1}\) indicated an anti-parallel organization of the strands (Figure 6.1 C, D). The β-sheet organization of lipopeptide 2 at the air-water interface was further confirmed by in situ grazing incidence X-ray diffraction (GIXD) measurements. GIXD investigations revealed the existence of diffraction peaks even before monolayer compression had started, thus indicating a 2D-crystalline structure. The presence of a Bragg peak corresponding to a distance d = 4.7-4.8 Å, both at low (i.e. Π = 1 mN/m) and high (i.e. Π = 30 mN/m) surface pressure, was attributed to the spacing of β-strands along the interstrand hydrogen bond direction. In the direction of the strand length, the spacing changed upon compression from ~ 64 to ~ 38 Å. The details of the GIXD measurements will not be further discussed here.

From the isotherm recorded on water a limiting mean molecular area of 189 Å\(^2\)/molecule was deduced by extrapolation of the slope in the liquid condensed region to zero pressure. This value correlates well with the value of ~ 200 Å\(^2\)/molecule estimated from combining the expected limiting Mma for the octapeptide segment (~ 130Å\(^2\)/molecule, from crystal structure data) and the experimentally determined limiting Mma of DOPE (71 Å\(^2\)/molecule). However, the arrangement of the two components with respect to each other has not been resolved yet.
Self-organizing $\beta$-sheet lipopeptide monolayers as template for the crystallization of CaCO$_3$

Figure 6.1 (A-B) $\Pi$-Mma isotherms of the monolayer of (A) lipopeptide 2 and (B) N-acetylated octapeptide 3 on H$_2$O (a), 10 mM CaCl$_2$ (b) and 9 mM Ca(HCO$_3$)$_2$ (c) sub-phase at 20 °C. The limiting molecular areas for compounds 2 and 3 were determined by extrapolating the slope in the liquid condensed region to zero pressure (see dashed line as an example for 3). (C) CD and (D) FT-IR spectra of the monolayer of lipopeptide 2 transferred from a water sub-phase at $\Pi = 30$ mN/m on quartz and ZnSe crystal, respectively at 20 °C.

Figure 6.2 BAM images of (A) uncompressed and (B) compressed monolayer of 2. Size of images: 400 x 300 $\mu$m$^2$.

In order to verify the stability of the lipopeptide secondary structure upon complexation with Ca$^{2+}$ ions, isotherms were recorded using an aqueous 10 mM CaCl$_2$ solution as the sub-phase (Figure 6.1 A). An expansion in limiting molecular area from 189 to 202 Å$^2$/molecule was observed, however, CD and IR spectra of the transferred monolayers still revealed the spectroscopic characteristics typical of $\beta$-pleated strands.\[40\] Also, in situ GIXD on CaCl$_2$ subphase (10 mM) showed that the 2D-crystallinity was retained. A further expansion of the molecular area to approximately 250 Å$^2$/molecule was observed when 2 was spread on a sub-phase containing 9 mM Ca(HCO$_3$)$_2$ (Figure 6.1 A), which is the solution used in the crystallization experiments described below.\[42\] These results indicate that, due to the DOPE
moiety, the lipopeptide 2 forms a monolayer that self-assembles without compression, but it is still dynamic and adapts its structure upon complexation of calcium ions to efficiently interact with the nucleating crystals.

![Proposed schematic representation of the β-sheet assemblies of 2 at the air-water interface.](image)

**Figure 6.3** Proposed schematic representation of the β-sheet assemblies of 2 at the air-water interface. The monolayer exposes an ordered array of carboxylic acid groups to the aqueous phase. (A) Top view. (B) Side view. Image generated using Materials Studio Version 2.0. [43] In (B) the phospholipid tails, which are probably randomly distributed in the hydrophobic region above the air-water interface, were left out for simplicity.

### 6.3 CaCO₃ crystallization under β-sheet lipopeptide monolayers

Calcium carbonate crystals were grown under self-organized monolayers of 2 (in crystallization dishes, without compression, Π ~ 30 mN/m) using the Kitano method [42] and collected after 5, 20 and 48 hrs. Calcite was formed predominantly in the form of pyramidal-shaped crystals together with rhombohedral crystals having a concave central region. [44]

![Pyramidal calcite crystals grown under monolayers of 2.](image)

**Figure 6.4** Pyramidal calcite crystals grown under monolayers of 2. (A-B) SEM image of a (A) (10.1) – oriented pyramidal crystal [(10.1) face belongs to the set {01.1}] [45] and of a (B) (01.2) – oriented pyramidal crystal viewed from the side exposed to the solution. (C) Shape Tm models [46] of the crystals shown in (A) – left and in (B) – right. (D) SEM image of a (01.2) – oriented pyramidal crystal as observed from the side attached to the monolayer.
The pyramidal shaped crystals were characterized by 3 thermodynamically stable \{10.4\} faces, which had been oriented to the aqueous phase, and one face consisting of 3 facets that had been oriented to the monolayer (Figure 6.4). Crystals showing a similar elevated feature consisting of three inclined facets have been observed before. \cite{13, 21, 47} It has been suggested that the apex represented the initial point of attachment to the monolayer and that the outer edges of the crystal detached in time from the monolayer due to gravity. Computer modeling of the pyramidal-shaped crystals, based on the scanning electron micrographs, \cite{46} indicated that in almost all cases the faceted faces belonged to the class \{01.\_l\}, with \_l = 1-2 (Figure 6.4). The formation of crystal faces of the type \{01.\_l\} with \_l = 1-2 in solution has been reported frequently for biogenic calcite \cite{48, 49} and recently also for the octapeptide (Phe-Asp)$_4$. \cite{20} In addition, (01.2) oriented calcite crystals have been obtained using different monolayer systems. \cite{11, 12, 14, 19, 48-50}

**Figure 6.5** Rhombohedral calcite crystals with a concave central region, grown under monolayers of 2. (A) Left: SEM image of a crystal with a concave central region viewed from the side attached to the monolayer and (top right) SEM image of a similar crystal viewed from the side exposed to the solution. Bottom right: Shape model of the crystal in the left image. \cite{46} (B) Left: SEM image of an indented crystal showing a central patch (highlighted). Right: TEM image and corresponding electron diffraction pattern of a rhombohedral crystal isolated after 20 min. The pattern corresponds to the [10.0] zone of calcite. Reflections A, \(\{01.1\}\) (4.2 Å); B, \(\{01.4\}\) (3.0 Å); C, \(\{00.5\}\) (3.4 Å). Angles, \(\{01.1\}\)\(\{00.5\}\) = 104°; \(\{01.1\}\)\(\{01.4\}\) = 59°. Camera length = 60 cm. Bars represent 20 μm except when indicated otherwise.
Previously, Lahiri et al. reported the formation of calcite crystals with a symmetrical indentation defined by three \( \{01.2\} \) faces around a \( \{00.1\} \) face which formed the attachment point to a porphyrin monolayer. \[51\] In the present case the concave indentation of the rhombohedral crystals is defined by 4 roughened planes (Figure 6.5), the outer edges of which form a plane that can be modeled by the \( (11.4) \) face of calcite. This morphological appearance of the indented rhombohedral crystals, however, has not been reported before. Also in the present case, on several crystals a central patch could be located (Figure 6.5 B). The indented crystals were dominant over the pyramidal crystals after 5, 20 and 48 hrs and this population slightly increased in time (Figure 6.6 A). Selected area electron diffraction performed on young crystals with a rhombohedral shape, collected within 20 min after the start of the experiment, revealed that these crystals had a \( \{10.0\} \) orientation (Figure 6.5 B). This suggests that for the indented crystals nucleation starts from a \( \{10.0\} \) face.

The observed crystal indentation might be the combined result of gravity and a limited \( \text{Ca}^{2+} \) and \( \text{HCO}_3^- \) ion transport from the mineralization solution. As the crystal grows it becomes heavier and tends to sink, while still being attached to the monolayer. Being flexible, the monolayer presumably bends to follow the sinking crystal. This will create a gap between the monolayer and the face of the crystal attached to it, towards the edges of the crystal (Figure 6.6 C). Therefore, the hindering effect of the monolayer on ion transport will be also reduced in that region. This will allow the outer corners of the crystal facing the monolayer to grow upwards, creating an indentation in the crystal.

**Figure 6.6 (A-B)** The distribution of the two populations of crystals (Type I – pyramidal shape, Type II – indented crystals) nucleated under monolayers of (A) lipopeptide 2 and (B) N-acetylated octapeptide 3, in percentage of the number of modified crystals (crystals isolated after 5, 20 and 48 hrs). (C) Proposed mechanism for the formation of the indented crystals.
For comparison, crystals were also grown under compressed monolayers of the lipopeptide 2 in a Langmuir trough. These experiments were performed at a surface pressure of 30 mN/m and yielded similar results as those performed using self-organizing monolayers spread in crystallization dishes. In addition a crystallization experiment in which the amount of lipopeptide 2 covered only 10 % of the available surface area was also performed. Under these conditions a similar ratio of pyramidal and indented crystals were obtained after 20 hrs, indicating that also at lower surface concentrations the monolayer self-assembled to attain the same template structure.

6.4 N-Acetylated octapeptide monolayers

For comparison the N-acetylated octapeptide 3 was also investigated. The Π-Mma isotherm of 3 revealed a sharp transition from a gas analogous to a 2D solid phase (Figure 6.1 B). From this curve a limiting mean molecular area of 61 Å²/molecule was determined, which was lower than the expected value, estimated from crystal structure data (130 Å²/molecule [41]), probably due to the high water solubility of 3. Nevertheless, CD and FT-IR studies, [35] performed on the transferred monolayers, indicated the presence of an anti-parallel β-sheet structure. [40] The CD spectrum displayed a maximum at 201 nm and a minimum at 221 nm, while the IR spectra showed a strong band at 1627 cm⁻¹ and a weak one at 1694 cm⁻¹. [52] When the isotherm of 3 was recorded on a 10 mM CaCl₂ sub-phase or on a sub-phase containing 9 mM Ca(HCO₃)₂ no expansion of the monolayer was observed, suggesting that 3, in contrast to the lipopeptide 2, does not significantly adapt its structure upon exposure to Ca²⁺ ions (Figure 6.1 B). One can speculate that it is likely that, in the case of 2, the presence of the unsaturated lipid chains slightly disturbs the crystallinity of the monolayer, thereby increasing its flexibility and its ability to adapt to the crystals nucleating underneath.

6.5 CaCO₃ crystallization under N-acetylated octapeptide monolayers

Crystals grown under monolayers of 3 again showed both types of modifications. However, in this case predominantly pyramidal crystals were observed after 5 hrs and only a minor (<5 %) amount of indented crystals was present. The latter population increased to approximately 50% of the total number of modified crystals after 20 hrs and only after 48 hrs became predominant (65 %, Figure 6.6 B). Importantly, most of the pyramidal crystals increased in size rather than in number, suggesting that their nucleation only occurred in the earlier stages of the experiment. In contrast, the appearance of small indented crystals was observed throughout the whole experiment indicating that these nucleated also in later stages of the experiment.

From the change of the composition of the mineral phase in time it becomes apparent that the monolayers of 2 and 3 differ in their ability to nucleate the indented vs. the pyramidal morphology at the different time points. It is proposed that in the case of 3 the nucleation of the face leading to the indented calcite takes place in later stages of the experiments, probably because the solid-like monolayer needs more time to reorganize and to adapt to the growing crystals.
6.6 Importance of template adaptability

The {01.1} and {01.2} crystal planes are defined by the same 4.99 Å distance in one direction, but show a gradual change in the orientation of the carbonate groups which are rotated over 13° going from \( l = 1 \) to \( l = 2 \) (Figure 6.7). The 4.99 Å distance most probably relates to the interstrand distance in the template as defined by the hydrogen bonds in the \( \beta \)-sheet (4.7-4.8 Å, GIXD [40]), allowing an approximate match of the template carboxylate groups with the carbonate ions in the crystal plane. However, in the other direction, although there is only 13° rotation between the planes with \( l = 1 \) and \( l = 2 \), significant differences are found in the inter-ion distances in the different nucleation planes. It is therefore likely that in this direction the alignment of the carboxylate groups with respect to the carbonate ions plays a much more important role than the matching of the distances of the two phases.

The more adaptable monolayer of 2 clearly favors the formation of a different set of planes, i.e. those belonging to the {10.0} family. Interestingly, these planes are related to the {01.l} planes (with \( l = 1-2 \)) by a further rotation of the carbonate ions by 14° when going from {01.1} to {10.0} (Figure 6.7). The {10.0} faces are again defined by a distance of 4.99 Å in one direction and have a spacing of 8.53 Å in the other direction. This suggests that also in this case the spacing of 4.99 Å relates to the interstrand distance in the \( \beta \)-sheet of 4.7-4.8 Å. The interaction of the carboxylate groups of the template with the {10.0} faces along the other direction is most likely related to the ability of the monolayer to adapt to the growing crystal.

Figure 6.7 Materials Studio ™ – generated model of the (A) (01.2), (B) (01.1) and (C) (10.0) plane of calcite with the viewing direction (left) along and (right) perpendicular to the plane. [43]

For this compound (2) the surface pressure–surface area isotherms recorded on H₂O and Ca(HCO₃)₂ sub-phases showed an expansion of the limiting mean molecular area from 189 to
Self-organizing β-sheet lipopeptide monolayers as template for the crystallization of CaCO₃

250 Å²/molecule, respectively. It is presumed that the expansion in the direction of the backbone of the molecule should account for most of the observed increase in mean molecular area. [53] If a small expansion in the interstrand distance from 4.7-4.8 Å to 4.99 Å is envisioned, the molecule should then expand from ~ 40 Å to ~ 50 Å (i.e. with ~ 25 %) along its long axis. It is important to note, however, that stretching of the peptide backbone alone to an all-trans configuration yields a maximum distance between the Glu side chains of ~ 7.6 Å compared to the 8.53 Å spacing of the {10.0} face. Nevertheless, the flexibility of the template should allow it to organize such that the carboxylates align with the carbonate ions in the nucleation plane while matching the 4.99 Å distance, similar as was proposed for amide-containing phospholipid derivatives. [13] However, the importance of other factors, such as charge density of the amphiphile and the possible presence of a layer of carbonate ions between the crystal and the monolayer should not be neglected. [18-21] It is proposed that the N-acetylated octapeptide 3 has a limited ability to adapt to the growing crystal and hence is responsible for the preferred nucleation of the {01.1} faces (with l = 1-2) and the much slower formation of the indented crystals.

6.7 Conclusion

In conclusion, the results presented in this chapter demonstrate that the amphiphilic lipopeptide 2 formed stable monolayers with an anti-parallel β-sheet conformation. This enabled not only the study of the effect of these monolayers on the crystallization of calcium carbonate, but also the investigation of the way they interacted with the developing mineral phase. Indeed the formation of habit-modified calcite was observed. Apart from a small amount of pyramidal {01.1} oriented crystals (l = 1-2), the majority of the modifications resulted in a new type of indented calcite crystals, which, according to electron diffraction studies, nucleated from a {10.0} face. The formation of this new morphological form was significantly suppressed, when the less adaptable peptide 3 was used.

In previous studies, the formation of both {01.2} [14, 48-50] and {10.0} [13, 54] oriented crystals has been related to geometrical lattice matching and stereochemical complementarity between the functional groups of the template and the positions of ions in the nucleation plane. In other studies the oriented nucleation of crystals was attributed to non-specific electrostatic effects rather than to an epitaxial match between the monolayer and the crystal face. [16-21] The present system demonstrates that the nucleation of different crystal faces can be achieved depending on the ability of the template to adapt to the structure of the inorganic phase. Remarkably, although the importance of the flexibility of the template has been known for many years, [7, 55-57] it is rarely taken into account in reports on crystal nucleation. Furthermore the present results indicate that stretching of the template in only one direction allows the reorientation of the template’s functional groups such that the stabilization of different crystal planes can be achieved without the need for an epitaxial relation between the two components.
6.8 Experimental section

Materials. The lipopeptide 2 and the N-acetylated octapeptide 3 were synthesized by Silvia Cavalli and Emily E. Tellers (Leiden University, The Netherlands). Chloroform (AR) and methanol (AR) were purchased from Biosolve Ltd., trifluoroacetic acid (TFA) from Aldrich and nitric acid 65 % (AR) from VWR. Calcium carbonate (precipitated, p.a.) was purchased from Merck. All reagents and solvents were used as received. Ultra pure water (18 MΩ cm) was generated using a Barnstead Easypure® LF water purification system.

Surface pressure – surface area (Π-Mma) isotherms. All isotherms were recorded in an integrated dust free cabinet at 20 ºC and ambient humidity. Monolayers were formed by spreading solutions (1 mg/mL) of 2 and 3, respectively, from CHCl3/TFA (9/1, v/v) on water or supersaturated Ca(HCO3)2 subphase (prepared as described in section Crystallization experiments) in a commercial Teflon® Langmuir trough (KSV Minitrough, KSV Instrument Ltd., Helsinki, Finland). The monolayer was left undisturbed for 15 minutes (to allow solvent evaporation) prior to compression. Computer-controlled symmetrically movable hydrophobic Delrin® (polyacetal) barriers were used to regulate the surface area. The trough dimensions were 260 mm x 75 mm x 5 mm. The surface pressure was measured by a Pt Wilhelmy plate with a sensitivity of ±0.01 mN/m. The compression rate was set at 5 or 10 mm/min. The clean subphase was taken as the zero reference. Each isotherm was recorded at least in duplo.

Brewster Angle Microscopy (BAM). For BAM measurements, a Brewster Angle Microscope (KSV Optrel BAM 300, KSV Instrument Ltd., Helsinki, Finland) was mounted on the Langmuir trough described above. The BAM was equipped with a 633 nm (10 mW) He-Ne laser and images were collected with a CCD camera (768x72 pixels) connected to a PC. A lens (working distance = 33.5 mm; magnification = 5; numerical aperture = 0.28) was employed to focus the image. The incident and reflective angles were precisely adjusted (accuracy = 0.01°) to the Brewster angle of water to attain minimal reflection at the air-water interface. A black non-reflective sheet was also placed on the base of the trough to minimize the reflection of the refracted beam. The captured images from the BAM were integrated with the data recorded for the surface pressure – surface area isotherm.

Crystallization experiments. All glassware used for crystallization experiments was thoroughly cleaned with soap, nitric acid solution (~ 10%, overnight) and methanol. Between each cleaning step, the glassware was rinsed extensively (3x) with ultrapure water. For all crystallization experiments a ~ 9 mM supersaturated solution of calcium bicarbonate, Ca(HCO3)2, prepared following the Kitano method, was used. The solution was made by bubbling CO2 gas through a suspension of CaCO3 (3.5-4 g) in ultrapure water (1.5 L) for 1 hr ½, followed by filtration and CO2 bubbling for another 30 min to dissolve any CaCO3 particles present. Monomolecular films of 2 and 3 were spread from CHCl3/TFA (9/1, v/v) solutions (1 mg/mL) at the air / water interface of freshly prepared supersaturated Ca(HCO3)2 solution poured in crystallization dishes (Schott Duran®, Germany) (50 mL/dish) or in the Teflon® Langmuir trough described above. For the crystallization dishes, the amount of molecules to spread was calculated taking into account the limiting Mma (obtained by extrapolating the surface pressure-area isotherms to zero pressure - 189 Å2/molecule for 2 and 61 Å2/molecule for 3) such that the molecules would cover 100 % and 10 % of the surface of the crystallization dish, respectively. For the experiments performed in the Langmuir trough, the monolayers were compressed until reaching a surface pressure Π = 30mN/m, which was maintained constant throughout the experiment. Crystallization of CaCO3 was governed by the slow loss of CO2 gas from the supersaturated solution according to equation (1). [57]

\[
\text{Ca}^{2+} (aq) + 2\text{HCO}_3^- (aq) \rightarrow \text{CaCO}_3 (s) + \text{CO}_2 (g) + \text{H}_2\text{O} (l)
\]  

Figure 6.8 Schematic representation of the experimental setup used for the growth of CaCO3 crystals under a monolayer template.
Crystals used for optical microscopy and SEM investigation were collected on glass microscopy slides (diameter = 15 mm, Menzel-Gläser, Germany) by vertical (Langmuir-Blodgett) and horizontal (Langmuir-Schaefer) transfer after 5, 24 and 48 hrs, following established procedures. The crystals isolated by vertical dipping of the glass slides through the monolayer expose the faces that were attached to the monolayer, while the crystals isolated by horizontal dipping expose the side that was facing the mineralization solution (Figure 6.9).

**Figure 6.9** Sample isolation from Langmuir monolayers. (A) Langmuir-Schaefer and (B) Langmuir-Blodgett dipping.

**Optical Microscopy.** See Chapter 5 (Experimental section).

**Scanning Electron Microscopy (SEM).** See Chapter 5 (Experimental section).

**Transmission Electron Microscopy (TEM).** TEM was performed on a JEOL 2000-FX electron microscope operating at an accelerating voltage of 80 or 120 kV. Crystals used for selected area electron diffraction were collected within 20 minutes after the start of the experiment by Langmuir-Schaefer transfer on carbon-coated TEM grids. The isolation procedure is based on an established technique, which mitigate against any disruption of the crystal alignment relative to the template. Therefore, the crystals isolated on the TEM grids should be laying on the face that was attached to the monolayer, i.e. the face they nucleated from.
6.9 References and notes

[35] CD and FT-IR characterization of monolayers of 2 and 3 transferred to quartz plates and ZnSe prisms, respectively, was performed by Silvia Cavalli and Emily E. Tellers (Leiden University, The Netherlands).
[36] Previous studies have demonstrated that the organization of similar peptides at the air-water interface was preserved upon transfer to solid supports for FT-IR investigations (see reference [33]).
[39] In situ GIHD measurements on water and CaCl2 solution (10 mM) sub-phase were done by Dr. H. Rapaport (Ben Gurion University of the Negev, Israel) and S. Cavalli (Leiden University, The Netherlands).
[41] The repeat distances of 4.7 and 6.9 Å, that have been observed previously in crystalline β-sheet structures can be used to estimate the area per molecule, e. g. 4 x 4.7 x 6.9 = 129.7 Å for an 8 residue peptide. See reference [33].
[43] Computer models were generated using Materials Studio™ Version 2.0, © 2001 by Accelrys Inc. (USA).
[44] In the absence of a monolayer only unmodified [10.4] calcite and some vaterite crystals were isolated.
[45] For a hexagonal space group, the symmetry related planes in a [hkkl] set are (hkkl), (−l+(h+k),l), (−l+(h+k),l,h), and their Friedel pairs. See J. Aizenberg, J. Hanson, T. F. Koetzle, S. Weiner, L. Addadi J. Am. Chem. Soc. 1997, 119, 881.
[46] Calcite crystals were modeled with SHAPE V7.1.2. ©2004 by Shape Software, Kingsport (USA).
Self-organizing β-sheet lipopeptide monolayers as template for the crystallization of CaCO₃

[52] In aqueous solution the acetylated octapeptide 3 showed a random coil conformation.
[53] In situ GIXD measurements on water and CaCl₂ solution sub-phase showed that the interstrand distance remained constant upon compression. The strand length however, appeared to have an elastic behavior, as upon compression the conformation of the backbone changed.
Chapter 7

Tuning the organization of CaCO₃ crystallization templates:
Self-organizing amino acid-based surfactants

Abstract

This chapter describes a template system for CaCO₃ crystallization based on Langmuir monolayers of bisurea amphiphiles with amino acid head groups. By changing the size of the amino acid side group R, it is possible to control the packing and the flexibility of the monolayers. Consequently, as the bulkiness of R increases going from glycine to alanine and valine, the molecules become less closely packed and the monolayer organization becomes less rigid. These parameters significantly influence the ability of the monolayer template to induce oriented nucleation of calcite, as well as the crystallographic orientation of the resulting crystals. As a consequence, the most well-organized, rigid, glycine-based monolayer acts as a poor template, leading to mainly non-specific crystal nucleation. In the flexible valine-based monolayer, the molecules can rearrange to minimize the geometrical and orientational lattice mismatch with the nucleating crystal face. Thus the interfacial tension of the system is reduced and the nucleation of uniformly {10.0} oriented calcite crystals is promoted. The results presented here show that the capability of a monolayer template to direct nucleation and growth of uniformly oriented crystals is related to its ability to adapt to the structure of the inorganic phase.
7.1 Introduction

In biomineralization, crystal nucleation and growth are directed by self-assembled structures, generally consisting of acidic polypeptides that display an ordered array of functional groups. Inspired by these processes, researchers have used self-organized monolayer systems to control the growth of oriented CaCO₃ crystals \textit{in vitro}. Examples of these systems include self-assembled monolayers of functionalized alkanethiols supported on Au or Ag substrates, \cite{3-9} polymeric Langmuir-Schaefer films of 10,12-pentacosadiyonic acid, \cite{10} monolayers of hydrogen-bonded molecular ribbons consisting of \textit{N},\textit{N}'-dioctadecyltriazine-2,4,6-triamine and a cyanuric acid derivative, \cite{11} amphiphilic calixarenes and resorcarenes, \cite{12-14} porphyrin monolayers, \cite{15, 16} as well as self-organized monolayers of amide-containing phospholipids. \cite{17} However, only a few of these systems offer the possibility to change the spacing and orientation of the functional groups in a controlled manner. \cite{4, 9, 12-14, 18, 19} Aizenberg et al. have developed a system based on self-assembled monolayers (SAMs) of alkanethiols in which the spacing, ordering and orientation of the same terminal group (e.g. CO₂⁻, SO₃⁻ and OH) could be varied by selecting different substrates (i.e. Au and Ag). \cite{4} Their results revealed that, for all thiols, calcite crystals with different orientations were obtained by changing from Au to Ag. In all cases the orientation of the functional groups in the SAMs matched the orientation of the carbonate ions in the nucleating crystal face, while a geometrical match between the lattices of the SAM and the crystal could not be found. Also Tremel et al. used SAMs of thiols with different substituents and showed that, together with the polarity of the substrate, the monolayer packing geometry had a significant influence on polymorph selection. \cite{9, 18, 19} Volkmer et al. investigated the crystallization of CaCO₃ under monolayers of amphiphilic calix[n]arene (n = 4, 8) and resor[4]arene monolayers displaying predefined arrangements of carboxylate head groups. \cite{12-14} However, even though they had different packing arrangements, (01.2) oriented crystals were obtained for all templates. From this, Volkmer et al. concluded that non-specific electrostatic interactions determined crystal orientation.

In order to obtain more insight into the relationship between template organization and crystal orientation, template systems with tunable properties (e.g. flexibility, spacing and orientation of functional groups) are required. In this chapter a template system is described based on Langmuir monolayers of bisurea amphiphiles, whose packing and flexibility can be changed in a controlled way. The amphiphiles consist of a dodecyl chain, a bisureidoheptylene unit and an amino acid head group (Figure 7.1 A). The carboxylate groups, which are characteristic for most of organic matrices involved in biomineralization, are present to induce nucleation of CaCO₃. \cite{2} Langmuir monolayers of urea derivatives are known to self-organize at the air-water interface by strong bifurcated hydrogen bonding between the urea units (Figure 7.1 B). \cite{20-24} Therefore, in one direction, the spacing of the bisurea amphiphiles 1 in the monolayer should be predetermined by the hydrogen bonding distances between the bisurea units. In the other direction, it is expected that the intermolecular distance is determined by steric interactions between the side groups R of the amino acid moiety (Figure 7.1B). From this it was anticipated that by choosing different amino acids, i.e. going from glycine to alanine to valine it should be possible to alter the packing density of the molecules and thereby modulate the distance between the functional groups in a systematic way. Changing the volume of the side group R also alters the flexibility of the monolayers and it will be discussed how these
two factors influence the ability of the template to induce oriented nucleation of calcite, as well as the crystallographic orientation of the resulting crystals.

![Figure 7.1 (A)](image)

**Figure 7.1 (A)** General structure of the bisurea-based amphiphiles used in this study. **(B)** Computer generated representation of an idealized model for the organization of the bisurea amphiphiles 1b in a monolayer at the air-water interface. **Left:** view normal to the direction of the hydrogen bonds. **Right:** view almost parallel to the direction of the hydrogen bonds. In this cartoon the molecules are presented without taking into account the degree of tilting of the monolayer with respect to the air-water interface.

## 7.2 Synthesis

The preparation of the bisurea amphiphiles 1 (Figure 7.1 A) was approached via the coupling of the monourea amine 2 to the different elected amino acids. The first step in the synthesis of 2 (Scheme 7.1) [25] involved the Boc-protection of one of the amine groups of 1,7-heptanediamine 4. The resulting compound 5 was then reacted with the dodecylisocyanate 7, which was prepared in situ from the corresponding amine 6 and di-\(t\)-butyltricarbonate, [26, 27] leading to the formation of 8. [28] Removal of the Boc-group of 8 using HCl yielded the desired intermediate 2.

Notably, the free acid form 1 of the bisurea amphiphiles (Figure 7.1 A) contains a hydantoic acid moiety that can cyclize under acidic conditions to form the hydantoin structure (Scheme 7.2). [29] Therefore, it was decided to prepare the target compounds as their corresponding K\(^+\) salts (11), which are expected to be more stable with respect to the formation of hydantoin.

To this end the isocyanate of the monourea amine 2 was prepared using the di-\(t\)-butyltricarbonate method (see above) and without work-up directly reacted with the three different amino acids (i.e. glycine, alanine and valine) using potassium hydroxide as the base (Scheme 7.3). [30, 31] This resulted in the formation of the target compounds (11a-c) which after purification by dialysis (11a,c) or precipitation from ethanol (11b) were isolated in moderate yields (30-55%).
Scheme 7.1 Synthetic route to the monourea amine 2.

[Chemical structure and reaction equations]

Scheme 7.2 Reaction mechanism of the acid-catalysed conversion of the hydantoin acid into the cyclic hydantoin structure.

[Chemical structure and reaction equations]

Scheme 7.3 Synthesis of the target bisurea amphiphiles.

[Chemical structure and reaction equations]
7.3 Monolayers of bisurea amphiphiles on water subphase

Surface pressure-surface area (Π-Mma) isotherms of the bisurea amphiphiles 11a-c were recorded on an aqueous subphase. The isotherms revealed in all cases a relatively small lift-off area (i.e. 34 Å²/molecule for 11a-b and 40 Å²/molecule for 11c - Table 7.1) after which the surface pressure increased steeply, indicating a transition into a 2D liquid-condensed state (Figure 7.2).

The absence of the liquid-expanded state in the isotherms of 11a-c was tentatively attributed to a pre-organization of the molecules into 2D aggregates driven by the hydrogen bonding propensity of the bisurea groups. Indeed, in situ Brewster Angle Microscopy (BAM) performed on monolayers of 11b revealed the presence of solid-like domains with dimensions in the millimeter regime (Figure 7.3). These were observed already in the gas analogous region of the isotherm confirming the self-organizing ability of this compound. In the early stages of compression (Π = 0 mN/m) these domains were clearly separated, however, upon further compression, the domains became connected and fused into a continuous film, with a concomitant steep increase of the surface pressure.

The limiting mean molecular areas (determined by extrapolation of the slope of the isotherms in the condensed phase to zero surface pressure) of 11a-c increased with the size of the side group R of the amino acid moiety (Table 7.1). This indicated that indeed the packing of the monolayer and thereby the 2D arrangement of the carboxylate groups was influenced by the volume of the side group. The compressibility of the monolayer of 11a was lower than for the monolayers of 11b and 11c (Table 7.1). This, together with the low limiting Mma points to the formation of a rigid, densely packed, pre-organized monolayer, as expected for glycine-based amphiphile 11a, which does not have a side group R that can interfere with the packing of the monolayer.
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**Figure 7.3** BAM images corresponding to the monolayer of 11b on water subphase (at 20 °C) (A) before compression (Π = 0 mN/m, Mma = 90 Å²/molecule) and (B-D) after compression has started at (B) Π = 0 mN/m, Mma = 59.1 Å²/molecule; (C) Π = 10.3 mN/m, Mma = 28.6 Å²/molecule and (D) Π = 54.8 mN/m, Mma = 15.8 Å²/molecule (after the collapse point). Scale bars = 100 μm.

**Table 7.1** Data extracted from the Π-Mma isotherms of 11a-c.

<table>
<thead>
<tr>
<th>Amphiphile</th>
<th>Side Group R</th>
<th>Limiting Mma (Å²/molecule)</th>
<th>Lift-off Mma (Å²/molecule)</th>
<th>Compressibility * x 10³ (m/mN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11a (Glycine-based)</td>
<td>-H</td>
<td>27</td>
<td>34</td>
<td>3.7</td>
</tr>
<tr>
<td>11b (Alanine-based)</td>
<td>-CH₃</td>
<td>30</td>
<td>34</td>
<td>6.1</td>
</tr>
<tr>
<td>11c (Valine-based)</td>
<td>-CH(CH₃)₂</td>
<td>34</td>
<td>40</td>
<td>5.6</td>
</tr>
</tbody>
</table>

* Compressibility = (-1/Mma)(dMma/dΠ) [33]

All monolayers exhibited high collapse pressures (50-70 mN/m), indicating that the bisurea amphiphiles formed stable monolayers at the air-water interface. To further investigate the stability of these monolayers, experiments were performed in which, at a fixed surface pressure Π = 40 mN/m, the surface area was monitored as a function of time (Figure 7.4). A reduction of surface area was observed, the extent of which increased going from 11a to 11b to 11c, but which was small in all cases (e.g. <5% over 30 min for 11c).
Figure 7.4 Reduction of the surface area as a function of time at constant pressure ($\Pi = 40$ mN/m) for the monolayers of (a) 11a (glycine-based), (b) 11b (alanine-based) and (c) 11c (valine-based). $A_0$ is the surface area at time $t = 0$ s, when $\Pi$ has reached 40 mN/m and $A_i$ is the surface area at time $t = i$ s.

When the monolayers of 11a-c were compressed to $\Pi = 40$-50 mN/m and subsequently allowed to expand a strong hysteresis effect was observed in all cases, showing that the aggregates formed by supramolecular interactions between the amphiphile molecules do not dissociate immediately when the layer is expanded. \[21, 32\]

Figure 7.5 (A-C) The compression and decompression isotherms of the monolayers of (A) 11a, (B) 11b and (C) 11c. (D) Variation of the limiting Mma of monolayers of 11a-c during compression-decompression studies.
The limiting Mma shifted further towards lower values in subsequent compression/expansion cycles (Figure 7.5). Significantly, the number of cycles required to achieve a constant limiting Mma increased with increasing the side group volume (Figure 7.5 D). This indicated that for surfactants with larger R-groups a more flexible monolayer was formed, which was able to rearrange and which reached its most efficient packing either upon standing or during compression-decompression cycles.

7.4 FT-IR Spectroscopy studies

In order to investigate the effect of the bulkiness of the side group R on the hydrogen bonding behavior of the amphiphiles, FT-IR spectra of monolayers of 11a-c were recorded in Reflection Absorption Spectroscopy (IRRAS) mode and compared to transmission spectra of films dropcast from chloroform/methanol solutions on ZnSe crystals. To obtain the IRRAS spectra monolayers of 11a-c were deposited on gold-coated glass substrates by Langmuir-Schaefer (horizontal) transfer (\( \Pi = 40 \text{ mN/m} \), Figure 7.6).

![Figure 7.6 Schematic representation of the Langmuir-Schaefer (horizontal) transfer of the monolayers of 11a-c from the air-water interface to gold-coated glass substrates.](image)

Importantly, IRRAS is only sensitive to vibrations that have a component oriented perpendicular to the substrate surface, the intensities of the vibrational modes parallel to the surface being largely suppressed. [21, 34] In the IRRAS spectra of the monolayers of 11a-b deposited on gold, both the N–H and C=O stretching vibrations were no longer present (Figure 7.7). The absence of these two vibration bands implies that the N–H and C=O bonds were oriented parallel to the gold substrate and thereby that hydrogen bonds between the bisurea units in the monolayers had been oriented parallel to the air-water interface (Figure 7.6). [21, 35] In the spectrum of 11c a small peak was still present at 1613 cm\(^{-1}\) (Figure 7.7), indicating that for
this compound the C=O bond vibration had a small component in the direction perpendicular to the substrate, and hence a tilted arrangement in the monolayer.

![Amide II absorption spectra](image1.png)

**Figure 7.7** IRRAS spectra of monolayers of 11a-c deposited by Langmuir-Schaefer transfer on gold-coated glass substrates. The N-H and C=O bands are not present. Only in the spectrum of 11c a small peak is visible at 1613 cm⁻¹, as indicated by the arrow.

IRRAS showed a shift of the amide II band to lower wavelengths with increasing size of the side group R (Figure 7.7), indicating a small decrease in the hydrogen bond strength going from 11a to 11b-c. [27, 36] This suggests that the amino acid side group does not only affect the packing of the surfactant molecules, but also their hydrogen bonding ability.

The FT-IR spectra of the dropcast films of 11a and 11c (Figure 7.8 and Table 7.2) showed similar values for the position of the amide II vibrations compared to the IRRAS spectra (1585 and 1574 cm⁻¹, respectively). For 11b, however, a shift was observed going from the monolayer (1576 cm⁻¹) to the dropcast film (1585 cm⁻¹). This difference was tentatively attributed to the possibility of the molecules of 11b in the dropcast film to form interdigitated bilayers in which the CH₃-group of the alanine moiety is not large enough to disturb the packing of neighbouring molecules in the same layer.

![FT-IR Transmission spectra](image2.png)

**Figure 7.8** FT-IR Transmission spectra of films of 11a-c dropcast on ZnSe crystals.
Although differences were observed in the IR spectra of the different compounds 11a-c, the \( \text{N–H stretch} \ (3331-3337 \ \text{cm}^{-1}) \) and \( \text{C=O stretch} \ (1611-1613 \ \text{cm}^{-1}) \) vibrations in the transmission spectra recorded from the dropcast films pointed to the formation of strong hydrogen bonds in all cases (Table 7.2). [27, 36, 37]

**Table 7.2** Wavenumbers of the N-H, C=O and amide II bands corresponding to dropcast films of 11a-c.

<table>
<thead>
<tr>
<th>Amphiphile</th>
<th>Vibration band (cm(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N-H stretching</td>
</tr>
<tr>
<td>11a (Glycine-based)</td>
<td>3331</td>
</tr>
<tr>
<td>11b (Alanine-based)</td>
<td>3331</td>
</tr>
<tr>
<td>11c (Valine-based)</td>
<td>3337</td>
</tr>
</tbody>
</table>

For all three amphiphiles the limiting \( \text{Mma} \) exceeded the molecular area corresponding to a close-packed hexagonal organization of alkyl chains (~ 20 Å\(^2\)/molecule). [38] This implies that in order to adopt a closely packed layer, the molecules of 11a-c must be tilted with respect to the normal to the air-water interface. In the IRRAS spectra, the intensity of the peak corresponding to the asymmetric CH\(_2\) stretching vibration band (~ 2927 cm\(^{-1}\)) is a measure for the tilt angle of the molecules with respect to the substrate. For closely packed molecules, oriented perfectly perpendicular to the air-water interface, the two C–H bonds in the CH\(_2\) groups of the alkyl chains should be laying in a plane parallel to the substrate [39] and the CH\(_2\) stretching bands should disappear in the IRRAS mode. Consequently, their presence in the IRRAS spectra of the monolayers of 11a-c (Figure 7.7) confirms the tilting of the molecules. It may be assumed that the molecules are tilted in a plane perpendicular to the direction of the hydrogen bonds, which should not be affected in this case. However, for the monolayers of 11b-c, a decrease in hydrogen bond strength was observed (compared to 11a), which was attributed to the influence of the volume of the side group \( R \). Therefore, for these monolayers, it cannot be excluded that the decrease is caused by a tilt of the molecules also in the direction of the intermolecular hydrogen bonds.

### 7.5 Crystallization of CaCO\(_3\)

Calcium carbonate crystallization underneath monolayers of 11a-c (\( \Pi = 40 \ \text{mN/m} \)) [40] using the Kitano method [41] resulted in the formation of rhombohedral single crystals of calcite. [42, 43] In the case of 11a, small fractions of vaterite and aragonite crystals were observed as well (< 5 %). The highest nucleation density was found under the more rigid glycine-based monolayer 11a (202 ± 16 crystals/mm\(^2\)) and the lowest for the most flexible valine-based monolayer 11c (48 ± 11 crystals/mm\(^2\)) (Table 7.3). However, the efficiency of the monolayer template in promoting oriented nucleation of crystals was high for 11c (70 % of the crystals had a uniform \{10.0\} orientation), low for 11b (only 15 % of the crystals were modified, with predominantly \{01.l\}, \( l = 1-2 \) orientations and a low fraction of \{10.0\} oriented crystals) and poor for 11a (10-15 % of the crystals were modified, but showed random orientations) (Table 7.3).
Table 7.3 Nucleation density, percentage of modified crystals and orientation of modified crystals obtained from crystallization experiments underneath monolayers of 11a-c (\(\Pi = 40\) mN/m). Crystals isolated after 4 hrs.

<table>
<thead>
<tr>
<th>Amphiphile</th>
<th>Nucleation density (crystals/mm²)</th>
<th>Modified * calcite crystals (%)</th>
<th>Orientation of modified crystals**</th>
</tr>
</thead>
<tbody>
<tr>
<td>11a (Glycine-based)</td>
<td>202 ± 16</td>
<td>10-15</td>
<td>Different orientations</td>
</tr>
<tr>
<td>11b (Alanine-based)</td>
<td>114 ± 19</td>
<td>15</td>
<td>{01.} with (l = 1)-2 (predominant)</td>
</tr>
<tr>
<td>11c (Valine-based)</td>
<td>48 ± 11</td>
<td>70</td>
<td>{10.0} (mainly nucleated in initial stages)</td>
</tr>
</tbody>
</table>

* The term “modified crystals” refers to crystals that have nucleated from a plane that does not belong to the \{10.4\} set. Rhombohedral calcite crystals showing only \{10.4\} faces are referred to as “unmodified crystals”.

** Crystallographic orientation at the monolayer/subphase interface.

**CaCO₃ crystallization under glycine-based monolayers (11a)**

SEM analysis of the modified calcite crystals isolated from underneath a monolayer of the glycine-based amphiphile after 4 hrs showed that these crystals had rhombohedral \{10.4\} faces and one roughened face that had been attached to the monolayer. SEM investigation further revealed that these crystals had different crystallographic orientations, implying that this monolayer had a poor templating effect. In order to probe the influence of Ca²⁺ ions on the organization of the monolayer of 11a, isotherms were recorded on an aqueous 9 mM CaCl₂ solution as well as on a supersaturated 9 mM calcium bicarbonate solution (Figure 7.9 A).[

![Graph](image)

**Figure 7.9** \(\Pi\)-Mma isotherms of the monolayers of 11a (glycine-based) on water subphase (solid line), 9 mM CaCl₂ subphase (pH = 8.6) (dash line) and 9 mM Ca(HCO₃)₂ subphase (pH = 5.6-6) (short dash line) at 20°C.

In both cases a small expansion in limiting Mma was observed compared to the isotherms recorded on water (Figure 7.9 D). However, the lift-off mean area was similar on all subphases,
suggesting that the overall organization of the monolayer did not significantly change due to the presence of Ca\(^{2+}\) ions. \cite{17} Most probably the rigid nature of the monolayer does not allow a reorganization of the molecules of \textit{11a} and thus the monolayer cannot adapt to a specific crystal face and promote oriented nucleation of crystals. The carboxylate groups of the monolayer accumulate calcium ions via non-specific electrostatic interactions, resulting in the formation of the thermodynamically most stable \{10.4\} calcite crystals (unmodified). The low fraction of modified crystals may have nucleated in few areas where the packing of the monolayer was not optimal and where the molecules had more freedom to rearrange. Since the modified crystals exhibited different orientations, it was not possible to determine any relationship between the monolayer and the crystals.

**CaCO\(_3\) crystallization under alanine-based monolayers (11b)**

For the alanine-based monolayer \textit{11b}, SEM analysis of the modified crystals isolated after 4 hrs revealed that these crystals consisted of rhombohedral \{10.4\} faces and one roughened face that had been attached to the monolayer, as for \textit{11a}. In this case, however, computer modeling of the SEM images indicated that for the majority of the modified crystals the roughened face belonged to the class \{01.\_\_\_\} with \(l = 1-2\) \cite{45} and for a small fraction (< 10 \% of the number of modified crystals) to the class \{10.0\} (Figure 7.10). \cite{46} Within the \{01.\_\_\_\} type, crystals with a \{01.2\} orientation were predominant.

![Figure 7.10 Top: SEM images of (01.2) (A, B) and (10.0) (C) oriented crystals grown under the monolayer of \textit{11b} isolated after (A, C) 4 hrs and (B) 20 hrs. Bottom: Shape \textsuperscript{TM} models \cite{46} of the crystals. Crystals viewed from the side that was exposed to the monolayer; the roughened faces have been attached to the monolayer (nucleation faces).](image)

Electron diffraction analysis of young crystals, isolated by Langmuir-Schaefer transfer on carbon coated TEM grids, revealed that after 7 min most of the modified crystals had a \{10.0\} orientation (Figure 7.11 A - inset). After 15 min, however, \{01.\_\} oriented crystals \((l = 1-2)\) were also present (Figure 7.11). Furthermore it was observed that in time the \{10.0\} oriented crystals increased in size rather than in number (Figure 7.10 and Figure 7.11). SEM analysis \cite{47} of
the crystals isolated after 15 min showed that at this stage the ratio of [10.0] oriented crystals to [01.\(l\)] oriented crystals was 1:2. For simplicity the crystals belonging to the class [01.\(l\)] were analyzed only as members of either the [01.1] type or the [01.2] type. After 15 min, the ratio of these two types was 1:1. However, after 4 and 20 hrs the [01.2] oriented crystals had become predominant over the [01.1] type crystals (as determined by computer modeling [46] of the crystals from the SEM images). Notably, the appearance of small [01.2] crystals was observed throughout the whole experiment (also after 20 hrs) (Figure 7.10 B). These results indicate that in the earlier stages of the experiment nucleation of the [10.0] face occurred whereas in later stages of the experiment [01.\(l\)] ([\(l\) = 1-2] nucleation became predominant, eventually favoring the [01.2] orientation.

![Figure 7.11](image)

**Figure 7.11** (A) [10.0], (B) [01.1] and (C) [01.2] oriented calcite crystals isolated from beneath a monolayer of 11b on a TEM grid by Langmuir-Schaefer transfer after 15 min. **Left**: SEM images. Scale bars: 1 \(\mu\)m. Crystals observed from the side exposed to the mineralization solution. **Right**: Shape\textsuperscript{TM} models [46] of calcite crystals viewed down perpendicular to the corresponding nucleating face: (10.0) (A), (01.1) (B) and (01.2) (C). Inset (A): Electron diffraction pattern corresponding to the [10.0] zone of calcite. Reflections A, (01.2) (3.9 Å); B, (01.6) (2.4 Å); C, (02.4) (1.9 Å). Angles, (01.2)\(^\wedge\)(01.6) = 84\(^\circ\); (01.2)\(^\wedge\)(02.4) = 54\(^\circ\). Camera length: 60 cm.

The fact that only a small fraction of the calcite crystals that nucleated under the monolayer of 11b were modified can be tentatively explained by the presence of regions with a
different degree of organization in the monolayer. This may give rise to a different nucleation behavior (Figure 7.12). More precisely, rigid, solid-like domains in which the molecules have limited mobility may be responsible for the formation of unmodified \{10.4\} calcite crystals, whereas the molecules in more “liquid”-like regions at the boundaries between the domains, may be able to reorganize such that they are able to template the formation of initially \{10.0\} and later \{01.1\}_{l} (l = 1-2) oriented crystals.

It has been shown before by other groups that a higher control of crystal orientation can be achieved under monolayers that can alter their organization during crystal growth to optimize the interaction between the template head groups and the crystal lattice. \[^{10,12}\] Also, it has been suggested that this process may occur more readily at the boundary between monolayer domains, where the molecules have more freedom of movement. \[^{48}\]

Figure 7.12 Schematic representation of the condensed and “liquid”-like regions in the monolayer of 11b.

The isotherm of 11b recorded on a CaCl\(_{2}\) subphase revealed a faster build-up in surface pressure compared to the one recorded on water (Figure 7.13 A). This effect is well documented for surfactants with carboxylate head groups and has been attributed to electrostatic and/or coordinative interactions with the Ca\(^{2+}\) ions in the subphase. \[^{13,15,49}\] However, upon changing to a Ca(HCO\(_{3}\))\(_{2}\) subphase a lower limiting molecular area was observed (Figure 7.13A). This suggests that changing the counter ion from Cl\(^{-}\) to HCO\(_{3}\)^{−} leads to the formation of a different type of complex.

Figure 7.13 (A) \(\Pi\)-Mma isotherms of the monolayers of 11b (alanine-based) bisurea amphiphile on water subphase (solid line), 9 mM CaCl\(_{2}\) subphase (pH = 8.6) (dash line) and 9 mM Ca(HCO\(_{3}\))\(_{2}\) subphase (pH = 5.6-6) (short dash line) at 20°C. (B) Proposed models for the organization of the monolayer of 11b in the presence of Ca\(^{2+}\) ions on 9 mM CaCl\(_{2}\) subphase (top) and on 9 mM Ca(HCO\(_{3}\))\(_{2}\) subphase (bottom).
This behavior may be explained by assuming the formation of a 1:1 complex between 11b and Ca\(^{2+}\) ions on a CaCl\(_2\) subphase with Cl\(^{-}\) ions interdigitating to provide charge neutrality (Figure 7.13 B). Upon changing to a Ca(HCO\(_3\))\(_2\) subphase the complex transforms into a 2:1 complex of 11b and Ca\(^{2+}\) that allows a closer packing of the surfactant molecules. Such a mechanism could also explain the formation of {10.0} oriented crystals in the early stages of mineralization as the nucleation of this neutral face, which consists of alternating calcium and carbonate ions, could be favored by the more expanded monolayer present in the stages prior to the rearrangement from a 1:1 to a 2:1 complex (Figure 7.14).\(^{[50]}\) The latter complex may account for the nucleation of the {01.2} oriented crystals, as it resembles this polar face of calcite that consists of a close packed layer of calcium ions. The limiting Mma of 34 Å\(^2\)/molecule observed on a Ca(HCO\(_3\))\(_2\) subphase is close to the unit cell area of the (01.2) face of calcite equal to 32 Å\(^2\) = 4.99 Å x 6.4 Å. The relationship between the organization of the template and the {01.1} face is not completely elucidated at the moment.

![Diagram](image)

**Figure 7.14** Proposed model for the reorganization of the “liquid”-like regions of the 11b monolayer on a supersaturated Ca(HCO\(_3\))\(_2\) solution during mineralization (left) and Materials Studio \(^\text{TM}\) generated models of the (10.0) and (01.2) faces of calcite.\(^{[51]}\)

{01.1} faces can be regarded as an intermediate between the {10.0} and {01.2} faces. The same crystals orientations (i.e. {01.1}, \(l = 1-2\) and (10.0)) were observed also for the peptide-based monolayers described in Chapter 6.\(^{[52]}\) It was discussed there that {10.0}, {01.1} and {01.2} crystal planes are defined by the same 4.99 Å distance in one direction, but show a gradual change in the orientation of the carbonate groups which are rotated by 14º going from {10.0} to {01.1} and further rotated by 13º, when going from {01.1} to {01.2} (see Chapter 6 – Figure 6.7). Also in the present case, the 4.99 Å distance is close to the 4.6 Å distance in the monolayer of 11b defined by the hydrogen bonds.\(^{[27, 53, 54]}\) However, in the other direction a relative match is only found for the (01.2) face, suggesting that in this direction the orientational match between the carboxylate groups in the monolayer and the carbonate ions may play a much more important role than the matching of the distances of the two phases.

**CaCO\(_3\) crystallization under valine-based monolayers (11c)**

SEM investigation of the modified crystals isolated from underneath monolayers of 11c after 4 hrs revealed the presence of uniformly habit-modified calcite, in which a rather large triangular face truncating the vertex of three intersecting {10.4} faces was observed (Figure 7.15
A). This face was characterized by a concave indentation defined in the present case by three roughened planes. Selective area electron diffraction performed on young crystals, isolated within 15 min from the beginning of the experiment, revealed that all these crystals had a {10.0} orientation, indicating that the crystals nucleated from this face (Figure 7.15 B-C). The formation of indented crystals has been reported before. Although sometimes a central patch was not clearly visible in mature crystals, as it is also the case in the present system, it has been suggested that crystal nucleation started in the center of the indentation, which was the initial point of attachment of the monolayer.

Figure 7.15 (A) SEM image of modified crystal with a concave indentation isolated from beneath a monolayer of 11c after 4 hrs. Crystal viewed from the side that was exposed to the monolayer. (B) TEM image of a young crystal isolated after 15 min and (C) its corresponding electron diffraction pattern. The pattern corresponds to the [10.0] zone of calcite. Reflections A, (00.6) (2.8 Å); B, (02.3) (2.0 Å); C, (02.3) (2.0 Å). Angles, (00.6)\(^\circ\) (0 2.3) = 111º; (00.6)\(^\circ\) (0 2.3) = 69º. Camera length: 60 cm.

Of all three compounds the change in lift-off Mma observed upon going from a water subphase to a subphase containing calcium ions was the most pronounced in the case of the valine-based amphiphile 11c (Figure 7.16 A). This suggests that the organization in this more flexible monolayer is more strongly affected by the complexation of calcium ions. For this compound however, the isotherms recorded on both calcium-containing solutions overlapped, indicating that the molecules adopted a similar organization on both subphases (Figure 7.16 B). This implies that, after complexation of Ca\(^{2+}\) ions, the template matches the [10.0] faces of calcite such that the monolayer does not need to further rearrange. Significantly, the spacing of 4.99 Å in the [10.0] faces of calcite may relate to the 4.6 Å distance between the head groups of the amphiphile molecules 11c in the direction of hydrogen bonds. The limiting Mma obtained on supersaturated Ca(HCO\(_3\))\(_2\) subphase was 37 Å\(^2\)/molecule = 4.6 Å x 8.0 Å, indicating that the average distance between the 11c molecules in the other direction is also close to the second spacing of 8.53 Å in the (10.0) face of calcite (Figure 7.17).
Chapter 7

Figure 7.16 (A) Π-Mma isotherms of the monolayers of 11c (valine-based) bisurea amphiphile on water subphase (solid line), 9 mM CaCl₂ subphase (pH = 8.6) (dash line) and 9 mM Ca(HCO₃)₂ subphase (pH = 5.6-6) (short dash line) at 20°C. (B) Proposed models for the organization of the monolayer of 11c in the presence of Ca²⁺ ions on 9 mM CaCl₂ subphase (top) and on 9 mM Ca(HCO₃)₂ subphase (bottom).

The fact that crystal indentation was only observed in the case of the more flexible valine-based monolayer (11c) and not for the glycine- (11a) and alanine-based (11b) monolayers supports the model proposed for the formation of indented crystals in Chapter 6 (see Figure 6.6 C). This model implies that the creation of the indentation is related to the flexibility of the monolayer and its ability to bend to follow the crystals, which once they grow and become heavier have the tendency to sink (for details see Chapter 6).

Figure 7.17 Computer generated model of the (10.0) face of calcite (top view). [51]

7.6 Conclusion

The results presented in this chapter demonstrate that by changing the size of the amino acid R group it is possible to control the packing and the flexibility of the monolayers of the bisurea amphiphiles. Consequently, as the bulkiness of R increases going from glycine to alanine and valine, the molecules are less closely packed and the monolayer organization becomes less rigid. These parameters significantly influence the ability of the monolayer template to induce oriented nucleation of calcite, as well as the orientation of the crystals (predominantly {01.₀}, l = 1-2 for the alanine-based monolayer and {10.₀} for the valine-based
monolayer). As a result, the most well-organized, rigid, glycine-based monolayer acts as a poor template, leading to mostly non-specific crystal nucleation. In the flexible valine-based monolayer, the molecules can rearrange to minimize the geometrical and orientational lattice mismatch with the nucleating crystal face. Thus the interfacial tension of the system is reduced and the nucleation of uniformly \(\{10.0\}\) oriented calcite crystals is promoted. \cite{10, 14, 57, 58} The inverse relationship between the oriented nucleation efficiency and the nucleation density (i.e. for 11c, a high percentage of \(\{10.0\}\) oriented crystals and a low nucleation density was observed) may be related to the fact that oriented nucleation requires the monolayer molecules to adopt an organization tailored for nucleation. Once the sites for the nucleation of \(\{10.0\}\) faces of calcite are formed, these sites compete against the formation of new nucleation centers on the monolayer such that nucleation is confined to a limited number of discrete sites across the monolayer surface. \cite{58} The results presented here support therefore the idea introduced in Chapter 6, i.e. the capability of a monolayer template to direct nucleation and growth of uniformly oriented crystals is related to its ability to adapt to the structure of the inorganic phase.
7.7 Experimental section

Materials. Unless otherwise stated, all reagents and chemicals were obtained from commercial sources and used without further purification. Dichloromethane (CH₂Cl₂) was freshly distilled over P₂O₅. Di-tert-butyl tricarbonate was prepared by B. F. M. de Waal (Laboratory of Macromolecular and Organic Chemistry, Eindhoven University of Technology) according to a literature procedure. [26, 59] See also Chapter 6 (Experimental section).

Synthesis

General methods. ¹H-NMR and ¹³C-NMR spectra were recorded on a 400 MHz NMR (Varian Mercury, 400 MHz for ¹H-NMR and 100 MHz for ¹³C-NMR) or a 300 MHz NMR (Varian Gemini, 300 MHz for ¹H-NMR and 75 MHz for ¹³C-NMR). For ¹H-NMR and ¹³C-NMR, chemical shifts are reported in ppm downfield from tetramethylsilane (TMS) and multiplicities as singlet (s), doublet (d), triplet (t) and multiplet (m). IR spectra were recorded on a Perkin Elmer 1600 FT-IR. Matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF) mass spectrometry has been performed on a PerSeptive Biosystems Voyager-DE PRO spectrometer. Elemental analysis has been carried out on a Perkin Elmer 2400. Compound 2 was synthesized following a procedure developed within the laboratory. [25]

(7-Amino-heptyl)-carbamic acid tert-butyl ester (5). Di-tert-butyl dicarbonate (16.67 g, 76.8 mmol) was dissolved in CH₂Cl₂ (160 mL), and added dropwise to a solution of 1,7-diaminoheptane (16.76 g, 115 mmol) in CH₂Cl₂ (90 mL). The suspension was stirred for 16 hrs at 20 °C. Subsequently, the reaction mixture was washed five times with water (150 mL) and the product was extracted by washing three times with aqueous 1 M HCl. The aqueous layers were removed and basified by addition of aqueous 10 M NaOH (100 mL) and subsequently extracted twice with CH₂Cl₂ (200 mL). The organic layers were combined, washed with water (5x, 100 mL) and dried over Na₂SO₄. The solvent was removed under reduced pressure and the product was obtained in a yield of 70 %. ¹H-NMR (CDCl₃): δ 4.55 (NHBOC, s, 1H), 3.08 (CH₃NHBOC, m, 2H), 2.65 (CH₂NH₂, t, 2H), 1.42 (CH₂, s, 9H), 1.29 (H₂NCH₂(CH₂)₂CH₂NH₂, m, 10H), 1.18 (H₂NCH₂, s, 2H). IR: 3342 (N-H stretching urea), 2962 (CH₃ asym. stretching), 2928 (CH₂ asym. stretching), 2856 (CH₂ symmetric stretching). 1688 (C=O urethane), 1526, 1390, 1364, 1274, 1249, 1170 cm⁻¹. MS (MALDI-TOF) m/z: 342.26 (M+H⁻). 1H and 13C-NMR spectrum [27, 28, 30, 50, 51].

[7-(3-Dodecyl-ureido)-heptyl]-carbamic acid tert-butyl ester (8). A solution of 1-dodecylamine (2.02 g, 10.9 mmol) in CH₂Cl₂ (25 mL) was quickly injected into a solution of di-tert-butyl tricarbonate (3.13 g, 11.94 mmol) in CH₂Cl₂ (25 mL), under vigorous stirring. After 60 min, 4 drops of pyridine were added to the solution. The mixture was then transferred to a solution of (7-amino-heptyl)-carbamic acid tert-butyl ester 5 (2.50 g, 10.9 mmol) in CH₂Cl₂ (12.5 mL), under stirring, upon which a suspension was formed, which was left stirring for 16 hrs. The target compound was isolated from the reaction mixture by column chromatography in a yield of 80 % (Silica gel, CH₂Cl₂/MeOH 97/3 v/v, Rf = 0.09). ¹H-NMR (CDCl₃): δ 4.57 (NHBOC, s, 1H), 4.41 (NHCONH₂, s, 2H), 3.15 (CH₂NH₂, m, 4H), 2.68 (CH₂NH₂, t, 2H), 1.48 (CH₂CH₂NH₂, m, 4H), 1.43 (CH₂CH₂NH₂, m, 2H) 1.32-1.25 (H(C₂H₅)₂CH₂, H₂NCH₂(CH₂)₅CH₂NH₂, m, 26H), 0.88 (CH₃, t, 3H). IR: 3382 (N-H stretching urethane), 3329 (N-H stretching urea), 2950 (CH₃ asymmetric stretching), 2916 (CH₂ symmetric stretching), 2852 (CH₂ symmetric stretching). 1689 (C=O urethane), 1614 (C=O urea), 1590 (amide II), 1514, 1388, 1365, 1320, 1269, 1173 cm⁻¹.

1-(7-Amino-heptyl)-3-dodecyl-urea (2). To a solution of [7-(3-dodecyl-ureido)-heptyl]-carbamic acid tert-butyl ester 8 (1.34 g, 3.03 mmol) in dichloromethane (18 mL) a 4M solution of HCl in dioxane (15 mL) was added. After 90 min the solvent was removed under reduced pressure. Solids were dispersed in CHCl₃ (40 mL) and aqueous 1M NaOH (75 mL) was added. The organic layer was isolated and, after addition of CHCl₃ (40 ml), it was washed with aqueous 1M NaOH solution (60 mL). The combined organic layers were dried over Na₂SO₄ and the solvent was removed under reduced pressure. The product was obtained in a yield of 70 %. ¹H-NMR (CDCl₃): δ 4.17 (NHCONH₂, s, 2H), 3.15 (CH₂NH₂, m, 4H), 2.68 (CH₂NH₂, t, 2H), 1.48 (CH₂CH₂NH₂, m, 4H), 1.43 (CH₂CH₂NH₂, m, 2H) 1.32-1.25 (H(C₂H₅)₂CH₂, H₂NCH₂(CH₂)₅CH₂NH₂, m, 26H), 0.88 (CH₃, t, 3H). ¹³C-NMR (CDCl₃): 159.2 (C=O), 41.4 (CH₂NH₂), 40.1 (CH₃NH₂), 40.0 (CH₂NH₂), 32.6, 31.8, 30.0, 29.9, 29.5, 29.5, 29.5, 29.2, 28.9, 26.8, 26.5, 26.5, 22.5, 13.9. IR: 3331 (N-H stretching urea), 2954 (CH₃ asymmetric stretching), 2920 (CH₂ asymmetric stretching), 2849 (CH₂ symmetric stretching), 1611 (C=O urea), 1576 (amide II), 1477, 1462, 1290, 1267, 1238 cm⁻¹. MS (MALDI-TOF) m/z: 342.26 (calculated [M+H⁺] 342.59). Elemental analysis: found - C(70.06 %), H(13.02 %), N(11.91 %); calculated - C(70.33 %), H(12.69 %), N(12.30 %).

[3-7-(3-Dodecyl-ureido)-heptyl-ureido]-acetic acid potassium salt (11a). Glycine (35.9 mg, 0.48 mmol) and KOH (34.3 mg, 0.611 mmol) were dissolved in water (2 mL). After water removal under reduced pressure, the solid
residue was redissolved in ethanol (2 mL). A solution of 1-{(7-amo-}
-heptyl)-3-dodecyl-urea 2 (146.8 mg, 0.43 mmol) in CHCl3 (13 mL) was
quickly injected into a solution of di-tert-butyl tricarbonate (124 mg, 0.47 mmol) in
CHCl3 (2.0 mL), under vigorous stirring. After 1.5 hrs, 4 drops of pyridine were added
and the solution was then transferred to the solution of glycine and KOH in ethanol under
stirring. Reaction was allowed to proceed for 16 hrs, after which the solvent was evaporated
under reduced pressure. The excess of amino acid and KOH was removed by dialysis
in water. To this end a suspension of the solids in DMSO (12 mL) and water (15 mL) was transferred to a dialysis
membrane (MWCO 12-14 kDa) and dialyzed for 3 days, during which the dialysate was refreshed with ultrapure
water every day. The water was removed from the aqueous suspension by freeze drying. After washing the freeze-
dried product with a CHCl3/MeOH (4/1 v/v) mixture, the desired compound was obtained as a white powder in
a yield of 30%. 1H-NMR (CDCl3/TFI-d 95/5 w/w): δ 3.95 (NHCH3COO), s, 2H), 3.13 (CH2NH, m, 6H), 1.52
(CH2CH2NH, m, 6H), 1.33-1.27, H3C(CH2)2CH2NH(CH2)2(CH2)2NH, m, 24H), 0.88 (CH2CH2, t, 3H). [60] IR:
3328 (N-H stretching), 2955 (CH3 asym. stretching), 2922 (CH2 asym. stretching), 2859 (CH2 sym. stretching), 1611
(C=O urea), 317 (NH asym. stretching), 297, 296, 295, 294, 292, 287, 286, 26.5, 26, 22.5, 19.2, 17.4, 17.0, 13.7. IR:
3337 (N-H stretching), 2958 (CH3 asym. stretching), 2922 (CH2 asym. stretching), 2850 (CH2 sym. stretching),
1614 (C=O urea), 1562 (amide II, C=O carboxylate), 1477, 1463, 1412, 1308, 1324, 1212 cm-1. MS (MALDI-TOF) m/z:
481.12 (calculated [M+H]+: 481.75). Elemental analysis: found – C(58.88 %), H(10.04 %), N(11.57 %); calculated
– C(58.26 %), H(9.57 %), N(11.32 %).

2-[3-{(3-dodecyl-ureido)-heptyl}-ureido]-propionic acid potassium salt (11b). Compound 11b was synthesized
according to the procedure described for 11a, using as reagents: L-alanine (40.2 mg, 0.45 mmol) and KOH (31.7 mg,
0.56 mmol), di-tert-butyl tricarbonate (130.2 mg, 0.50 mmol) and 1-(7-amino-heptyl)-3-dodecyl-urea 2 (154.2 mg,
0.45 mmol). After completion of the reaction, the solids were dispersed in ethanol (25 mL) after which, upon
heating above 70 °C, a homogeneous solution was obtained. The solution subsequently was slowly cooled down
to 4 °C, the resulting suspension was filtered and the residue, containing the pure product, was isolated (white
powder, yield 55 %). 1H-NMR (CDCl3/HFIP [62] -d2 95/5 w/w): δ 3.86 (CH2CH3, q, 1H), 3.07 (CH2NH, m, 6H), 1.48
(CH2CH2NH, m, 6H). 1H-NMR (CDCl3/CD3OD 95/5 w/w): 1.29-1.26, H3C(CH2)2CH2HN(CH2)2(CH2)2NH(CH2)2NH, m, 27H), 0.88 (CH2CH2, t, 3H).
13C-NMR (CDCl3/HFIP-d2 95/5 w/w): 182.7 (C=O acid) 159.4 (C=O urea) 159.3 (C=O urea), 52.0 (C=HCH3), 40.5
(CH2NH), 40.2 (CH2NH), 32.0, 32.0, 29.7, 29.7, 29.6, 29.5, 29.4, 29.4, 29.3, 29.2, 28.4, 26.8, 26.2, 26.1,
22.8, 19.2, 17.0, 14.1. IR: 3323 (N-H stretching), 2956 (CH3 asym. stretching), 2922 (CH2 asym. stretching), 2850 (CH2
sym. stretching), 1614 (C=O urea), 1562 (amide II, C=O carboxylate), 1477, 1463, 1412, 1308, 1260, 1241 cm-1.
MS (MALDI-TOF) m/z: 495.17 (calculated [M+H]+: 495.78). Elemental analysis: found – C(57.88 %), H(10.09 %),
N(10.99 %); calculated – C(58.26 %), H(9.57 %), N(11.32 %).

2-[3-{(3-dodecyl-ureido)-heptyl}-ureido]-3-methyl-butyric acid potassium salt (11c). The compound 11c was
prepared following the procedure described for 11a, using as reagents: L-valine (48.7 mg, 0.42 mmol), KOH (23.3
mg, 0.42 mmol), di-tert-butyl tricarbonate (121 mg, 0.46 mmol) and 1-(7-amino-heptyl)-3-dodecyl-urea 2 (141.9 mg,
0.42 mmol). For dialysis, the solids were dissolved in a minimal amount of EtOH to obtain a homogeneous solution
( ~ 5 mL) to which water (15 mL) was added, resulting in an aggregation of the product mixture. After freeze
drying, the product was obtained as a white powder (yield 55 %). 1H-NMR (CDCl3/CD3OD 90/10 v/v): δ 4.00
(CH2CH(CH3)2, d, 1H), 3.11 (CH2NH, m, 6H), 1.47 (CH2CH2NH, m, 6H), 1.23-1.27, H3C(CH2)2CH2HN(CH2)2(CH2)2NH, CHCH(CH3)2, m, 25H), 0.98 (CH2CH2, d, 3H), 0.92 (CH2CH2, d, 3H), 0.89 (CH2CH2, t, 3H), 13C-NMR (CDCl3): 177.8 (C=O acid) 159.6 (C=O urea) 159.4 (C=O urea), 60.0 (CH2CH(CH3)2), 39.9 (C=H2CH3), 39.8
(CH2NH), 39.7 (CH2NH), 31.7, 30.8, 30.0, 29.7, 29.7, 29.5, 29.4, 29.4, 29.2, 29.2, 28.7, 26.7, 26.5, 26.4, 22.5,
19.2, 17.4, 13.7. IR: 3337 (N-H stretching), 2958 (CH3 asym. stretching), 2922 (CH2 asym. stretching), 2849 (CH2 sym.
stretching), 1689 (C=O urea non-H bonded), 1615 (C=O urea H bonded), 1568 (amide II, C=O carboxylate), 1478,
1464, 1403, 1372, 1309, 1265, 1240, 1212 cm-1. MS (MALDI-TOF) m/z: 523.42 (calculated [M+H]+: 523.83). Elemental
analysis: found – C(61.12 %), H(10.32 %), N(11.05 %); calculated (C26H51.3N4O4K0.7) [61] – C(61.08 %), H(10.11 %),
N(11.89 %).
Monolayers

**Surface pressure-surface area isotherms.** See Chapter 6 (Experimental section).

<table>
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<tr>
<th>Table 7.4 Solutions used for Langmuir experiments</th>
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<tr>
<td><strong>Compound</strong></td>
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<td>11a</td>
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<td>11b</td>
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*Brewster Angle Microscopy.* See Chapter 6 (Experimental section).

**Infrared spectroscopy.** FT-IR measurements were done using a Biorad FTS6000 FTIR spectrometer, equipped with a DTGS detector. The spectra were recorded with a resolution of 4 cm⁻¹ and co-adding 1500 scans. For the reflection measurements (IRRA) a Harrick Seagul accessory was used. The reflection angle was 80°. P-polarised radiation was obtained using a rotatable wire grid polariser. Transmission spectra were obtained from samples cast on a ZnSe window from the CHCl₃/MeOH solutions used for Langmuir monolayer experiments.

**Crystallization experiments**

See also Chapter 6 (Experimental section). Crystallization experiments were performed in the Langmuir trough (at Π = 40 mN/m) and in crystallization dishes. In the later case the amount of molecules to spread was calculated taking into account the limiting Mma (obtained by extrapolating the surface pressure-area isotherms to zero pressure - 27 Å²/molecule for 11a, 30 Å²/molecule for 11b and 34 Å²/molecule for 11c) such that the molecules would cover ~100% of the surface of the crystallization dish. Crystals were isolated after 4 and 20 hrs on glass microscopy slides (both from the Langmuir trough and the crystallization dishes) and after 7 and 15 min on carbon-coated TEM grids (only from the crystallization dishes.)

**Scanning Electron Microscopy (SEM).** See Chapter 5 (Experimental section).

**Transmission Electron Microscopy (TEM).** See Chapter 6 (Experimental section).
7.8 References and notes


[25] Compound 2 was obtained following a synthetic route developed by N. Chebotareva, Eindhoven University of Technology, (unpublished results).


[28] The extent of the reaction leading to the formation of 8 was easily followed by monitoring the decrease of the peak corresponding to the isocyanate functionality by infrared spectroscopy.


[40] Crystallization experiments were performed for comparison both in the Langmuir trough (at Π = 40 mN/m) and in crystallization dishes in which ~100% of the surface was covered with molecules. Similar results were obtained in both experimental set-ups.


[42] In the absence of a monolayer only unmodified {10.4} calcite and some vaterite crystals were isolated.

[43] The presence of calcite was confirmed by Powder X-Ray Diffraction.

[44] The supersaturated Ca(HCO₃)₂ solution (9 mM) is the solution used in the mineralization experiments and it was prepared according to the Kitano procedure (for details see Chapter 6 - Experimental section).

[45] Crystals of the {01.1}, f = 1-2 class are common in biogenic minerals and include, besides the {01.1} and {01.2} sets also {02.3}, {03.4}, {05.6} etc. (see S. Albeck, J. Aizenberg, L. Addadi, S. Weiner, *J. Am. Chem. Soc.* 1993, 115, 11691).

[46] Calcite crystals were modeled with SHAPE V7.1.2. ©2004 by Shape Software, Kingsport (USA).

[47] The crystals were viewed down the surface normal and the three angles (α, β, γ) between the crystal edges meeting at the upper corner of the crystal (Figure 7.11 A) were measured. These angles can be...


[51] Computer models were generated using Materials Studio™ Version 2.0, © 2001 by Accelrys Inc. (USA).

[52] Several groups have reported the formation of [01\(l\)] \((l = 1-2)\) and [10\(0\)] oriented crystals using different templates. References to these works can be found in Chapter 6.


[54] Single-crystal X-Ray structure of a biphenylbisurea compound containing a bisureido-heptylene unit revealed a spacing between two hydrogen-bonded urea groups of 4.6 Å (see reference [53]). This is also comparable to the distance in crystal structures of other substituted urea groups described in the literature (see also a) A. Gesquiere, M. M. S. Abdel-Motattleb, S. De Feyter, F. C. De Schryver, R. Lazzaroni, J. L. Bredas, *Langmuir* 2000, 16, 10385; b) J. van Esch, S. De Feyter, R. M. Kellogg, F. C. De Schryver, B. L. Feringa, *Chem. Eur. J.* 1997, 3, 1238.)


[57] Due to the poor solubility of compound 11a, a complete \(^{13}\)C-NMR spectrum could not be obtained.

[58] In the FT-IR spectra of 11a and 11c, a small peak corresponding to the free carboxylic acid is also observed around 1730 cm\(^{-1}\).

[59] HFIP - Hexafluoroisopropanol
Chapter 8

Bisurea-based supramolecular templates for CaCO₃ crystallization

Abstract

This chapter describes the use of films of a bisurea-based thermoplastic elastomer (pTHF-U₄U), surface-modified by addition of an amine-functionalized bisurea molecule (2), as templates for CaCO₃ crystallization, resulting in the specific nucleation of the vaterite polymorph and control of the crystallographic orientation of the crystals formed. Characterization of the films by AFM, XPS and contact angle measurements shows that the surface of the polymer film containing more than 10 mol% of 2 is completely covered with aggregates of the additive molecules. From XPS and contact angle measurements it is concluded that, in the as-prepared films, the alkyl chains of the additive molecules are exposed at the surface. However, annealing in water at 50 °C induces a reconstruction of the surface of the modified polymer film, resulting in the formation of well-organized ribbon-like aggregates of 2 at the surface. These aggregates expose an array of NH₃⁺ functional groups, which induces the formation of oriented vaterite crystals on the surface-modified polymer films.
8.1 Introduction

The biomimetic synthesis of calcium carbonate in the presence of insoluble polymer matrices has been investigated with the aim of obtaining either model systems (to understand the mechanisms of control in biomineralization) or composite biomaterials with superior material properties. In biomineralization, control over crystallization is achieved through the combined effects of an insoluble polymer matrix that defines the nucleation event and soluble biomacromolecules that control crystal growth. Many of these control macromolecules contain domains rich in aspartic acid and polysaccharides, which are generally sulfonated or carboxylated. The oriented nucleation of calcium carbonate on a substrate requires a well-organized array of functional groups at the surface. It has been shown for several systems that the ability of the aspartic acid rich proteins to induce oriented nucleation was related to their organization into a β-sheet conformation after adsorption on the substrate. Falini et al. induced the formation of oriented vaterite and aragonite crystals using collagenous matrices with entrapped poly-L-aspartate (pAsp). Crystal orientation was believed to be controlled by the β-sheet structure of pAsp. Addadi et al. investigated the cooperative action of sulfonate and carboxylate groups on crystallization of CaCO₃ using substrates consisting of acidic macromolecules adsorbed on sulfonated polystyrene films. Oriented nucleation of calcite was observed only in the case of the aspartic acid rich proteins and pAsp, which adopted an ordered β-sheet conformation when adsorbed on the sulfonated polystyrene films. Moreover, both the sulfonate and carboxylate groups were required to induce oriented nucleation. Continuous thin films of CaCO₃ have been deposited on chitin, chitosan and poly(vinyl alcohol) matrices in the presence of polyelectrolytes such as poly(aspartic acid), poly(glutamic acid) and poly(acrylic acid). Kato et al. proposed a mechanism of mineral film formation based on the dual role of the soluble macromolecules which acted as inhibitors of the crystallization in solution, while promoting nucleation and growth on the substrate. In related studies, different polymorphs of CaCO₃ were deposited on biologically derived fibers, such as fibrin, chitin, cellulose, and silk in the absence and in the presence of polyelectrolytes. Synthetic materials (prepared using 6-methacrylamidohexanoic acid monomers and divinylbenzene as crosslinker), imprinted with motifs of crystal surfaces, have also been used to template the growth of specific crystal phases. In addition, polymer membranes with sponge-like structure (produced as templates of sea urchin skeletal plate) were used to obtain, in a constrained volume, calcite single crystals with a complex structure.

In this chapter the use of bisurea-based thermoplastic elastomers, surface-modified by addition of molecules containing a complementary bisurea unit and a functional group, as templates for CaCO₃ crystallization is described for the first time.

It is known that urea groups associate strongly via bifurcated hydrogen bonds. Bisurea-based materials have been investigated by several groups for different applications. The strong association by hydrogen bonding between low molecular weight compounds containing bisurea groups has been exploited to develop gelling agents. The properties of the gels could be varied by the use of different spacers (e.g. linear, cyclic or aromatic spacers) between the two urea groups. Moreover it has been shown that these bis-ureido units could be used to enantioselectively incorporate guest molecules via chiral recognition. The interest in (co)polymers based on the bisurea moiety has also been increasing in the last years due to their good mechanical properties. More recently, the concept of molecular
recognition has been demonstrated for thermoplastic elastomers having bisurea units as hard blocks, in which small molecules bearing complementary bisurea recognition units can be incorporated. [17, 25] In this way, modification of polymer properties or functionalization can be achieved using a modular approach, i.e. by mixing in complementary guest molecules with the desired functional groups.

In addition, self-assembling bisurea-based templates have been used to structure inorganic materials such as silica (e.g. silylated bisurea based compounds having different spacers between the bisurea groups: dodecylene, [26] cyclohexylene [27] and phenylene [28]) Also, the growth of CaCO₃ crystals in a bisurea dicarboxylic acid hydrogel has been studied as a model system to achieve a better understanding of the biomineralization process. [29, 30]

\[ \text{Scheme 8.1 Structures of the molecules used in this study.} \]

The bisurea thermoplastic elastomer chosen for the present study was a block copoly(ether)urea, pTHF-U₄U 1 (Scheme 8.1), consisting of poly(tetrahydrofuran) soft segments and bisureido-butylene hard segments. [17, 23, 25] Koevoets et al. have recently shown that the mechanical properties of this block copoly(ether)urea could be modulated by incorporation of low molecular weight bisurea guests, having the same distance between the urea groups as the polymer host. Also when bisurea containing dye molecules were mixed within the polymer film, strong discrimination between guests with matching and non-matching bisurea units was observed, resulting in different extractabilities of the dyes by detergent solutions. [25]

However it was also shown that, above a certain concentration of guest molecules mixed in, these could phase separate at the surface of the polymer film. [31] Therefore, it should be possible in principle, to completely cover the polymer film with a layer of functionalized bisurea-based molecules. The aim of the present study was to explore the surface-modification of films of the pTHF-U₄U thermoplastic elastomer by addition of the amine-functionalized bisurea compound 2, Scheme 8.1 (which has been shown to self-organize into ribbon like aggregates in aqueous solutions [32]) and also to examine the application of these films as templates for controlled CaCO₃ crystallization. The conditions required for coating the entire surface of the polymer film with molecules of 2, the availability of the functional groups, as well as the organization of the molecules at the surface of the film were investigated.

The exploitation of bisurea-based thermoplastic elastomers surface-modified by addition of functionalized bisurea-based molecules, as templates for CaCO₃ crystallization may be argued in the following way: (1) first, the strong association between the bisurea blocks via hydrogen bonding should provide a well-organized array of functional groups that may induce the oriented nucleation of crystals; (2) second, by addition of molecules with different functional groups (e.g. COO⁻, NH₃⁺ etc.) different polymorphs of CaCO₃ may be obtained [33] resulting in materials with different properties (such as mechanical properties and biodegradability); [2] (3) third, the processability of the thermoplastic elastomers [25] can be exploited to obtain porous 3D scaffolds (e.g. meshes of organized functionalized bisurea molecules supported on polymer fibers) for applications in bone tissue engineering.
8.2 Characterization of bisurea polymer films containing different amounts of additive molecules

Thin films of neat pTHF-U4U and pTHF-U4U containing different amounts of 2 (0.5, 2, 10, and 24 mol% with respect to the bisurea block of the polymer) were prepared by dropcasting 5 mg/mL polymer or polymer – additive 2 solutions in CHCl3/MeOH on glass substrates. Tapping Mode AFM investigation of the pTHF-U4U films revealed changes in the morphology of the films upon mixing-in various amounts of 2 (Figure 8.1). The morphology of the unmodified pTHF-U4U film (i.e. containing no additive 2) consisted of long fibers that were interpreted as hydrogen-bonded bisurea hard blocks (bright domains in the phase image) embedded in a matrix of the soft pTHF block (dark domains in the phase image) (Figure 8.1 A). This morphology is in agreement with previous reports. \cite{17, 25} The films containing 0.5 mol% additive molecules also showed a microphase separated fiber-like morphology, similar to the one of the unmodified polymer films (Figure 8.1 B). However, at 2 mol% additive molecules mixed-in, the appearance of large bright domains in the phase image was observed, which was attributed to macrophase separated domains of additive molecules 2 at the surface of the polymer film (Figure 8.1 C). In between the large bright regions a fiber morphology alike the morphology of the pTHF-U4U polymer film was still visible (Figure 8.1 D). The surface of the films containing 10 mol% of 2 was almost entirely coated with additive molecules which aggregated into elongated structures (Figure 8.1 E, G). There were only a few small regions where a microphase separated fiber morphology similar to the one of the unmodified pTHF-U4U film was still visible in between the aggregates of the additive molecules (Figure 8.1 E-F). The surface of the films with 24 mol% molecules of 2 mixed in was entirely coated with aggregates of 2 (Figure 8.1 H).

The macrophase separation of the additive molecules at the surface of the polymer film, suggests that these molecules were not incorporated within the pTHF-U4U hard blocks. Moreover, a fiber morphology similar to the morphology of the pTHF-U4U polymer film would be expected if all the bisurea units of the additive molecules were intercalated between complementary bisurea units of pTHF-U4U via hydrogen bonding. \cite{34}

Water contact angle (θ) measurements indicated that upon increasing the amount of additive molecules 2 mixed in 1, the polymer films became more hydrophobic. Upon incorporation of 0.5 up to 10 mol% additive molecules in the polymer films, a gradual increase in θ from 73±2° to 95±2° was recorded; for the polymer films containing 10 and 24 mol% additive molecules, θ did not increase further (Figure 8.2). These results are in agreement with the AFM investigations, which showed that for the films containing 10 and 24 mol% of 2, the surface was coated with additive molecules and the morphology of the films did not differ significantly.
The large contact angles with water (95±5º) recorded for the films containing 10 and 24 mol% additive molecules suggest that the hydrophobic alkyl chains and not the polar NH$_3^+$ groups of the amphiphile 2 were exposed to air.

The large contact angles with water (95±5º) recorded for the films containing 10 and 24 mol% additive molecules suggest that the hydrophobic alkyl chains and not the polar NH$_3^+$ groups of the amphiphile 2 were exposed to air.

Figure 8.2 Dependence of water contact angles of the polymer films on the amount of 2 added.

Figure 8.1 (A-H) Tapping Mode AFM phase images of pTHF-UiU films containing different amounts of additive molecule 2: 0 mol% (A), 0.5 mol% (B), 2 mol% (C-D), 10 mol% (E-G) and 24 mol% (H). (I) AFM phase image of the aggregates formed by 2 when cast on glass from a CHCl$_3$/MeOH solution. Phase shift: 10º (A), 15º (B), 50º (C, D), 25º (F, H, I) and 40º (E, G).
In order to probe the chemical composition of the upper 7-10 nm of the surface-modified polymer films containing 24 mol% of 2, XPS spectra were acquired at 0° with respect to the surface normal. The data showed that, the chemical composition of the polymer film surface was different from the bulk composition of the modified polymer film (calculated assuming the additive molecule was uniformly distributed within the film) (Figure 8.3 A). However, the experimental data were very close to the chemical composition calculated assuming that the entire surface of the film was covered only with molecules of 2, confirming therefore the previous AFM and contact angle measurements that pointed to an accumulation of 2 at the surface of the polymer film (Figure 8.3 A). The increase of the measured C1s signal corresponding to the alkyl chain of 2 (C-C) compared to the calculated composition suggested an enrichment in alkyl chains at the outermost surface of the polymer film.

**Figure 8.3** The calculated and experimentally determined atomic percentages of various atoms in the surface-modified polymer film, containing 24 mol% of additive molecules. Both the atomic percentages corresponding to a bulk film composition calculated assuming the additive molecule was uniformly distributed within the polymer film and the atomic percentages calculated assuming that only 2 was present at the surface are shown. Measured % is based on XPS spectra acquired at 0° (A) and 60° (B) with respect to the surface normal.

For XPS spectra acquired at 60° (probing thickness ~ 3 nm), the increase of the C1s signal corresponding to the alkyl chains (C-C) was even more pronounced (Figure 8.3 B). That indicates that indeed the molecules of 2 were oriented such that the alkyl chains were exposed at the film surface, supporting the contact angle measurements.

The results presented so far suggest that, when casting the modified polymer films from CHCl₃/MeOH solution, part of the additive molecules mixed in assemble at the surface of the film and form elongated aggregates (Figure 8.1 G, H) which expose the alkyl chains and have the NH₃⁺ groups buried inside. The morphology of these aggregates resembles the morphology of the aggregates formed by 2 alone when cast from a CHCl₃ / MeOH solution onto glass (Figure 8.1 I).

### 8.3 Rearrangement of the surface of the modified bisurea polymer films upon exposure to water

AFM, contact angle and XPS investigations showed that the additive molecules were present at the surface of the modified films. For 10 and 24 mol% additive molecules mixed in,
the surface of the films was (almost) completely coated with aggregates of 2. As the XPS and contact angle measurements indicated that the alkyl chains of the additive molecule 2 were exposed at the surface, it follows that the functional amine groups were buried inside the film. However, in order to achieve control over CaCO₃ crystal nucleation and growth on the surface-modified polymer film, the amine groups of the additive molecules need to be available at the surface. For amphiphilic block copolymers, it is known that, upon exposure to water, the hydrophilic domains migrate towards the surface in order to reduce the free energy of the water/polymer interface. [36, 37] From this it was anticipated that the surface of the modified polymer film, consisting of aggregates of the amphiphilic additive molecules, could possibly reorganize in contact with water.

Contact angle measurements of a water drop in contact with a dry surface-modified polymer film (containing 24 mol% of 2) revealed a decrease in contact angle (θ) of 10° within 120 s. [38] In the case of the neat pTHF-U₄U film, only a decrease of 4° was observed (Figure 8.4). [39] These results suggest that some reorganization of the surface of the modified polymer film occurs upon exposure to water. The additive molecules present at the surface probably reorganize to expose the polar NH₃⁺ groups towards water in order to reduce the interfacial energy.

![Figure 8.4](image_url) Variation of the contact angle with a water drop (θ) of the unmodified and surface-modified pTHF-U₄U films (containing 24 mol% additive molecule) during the first 2 min of contact with water. Δθ = θᵢ - θ₀ (where θ₀ represents the initial contact angle value at time, t = 0 s and θᵢ is the value at t = i s).

In order to further investigate the reorganization of the additive molecules at the surface of the modified polymer film containing 24 mol% of additive molecules, contact angle measurements using the captive air bubble method were performed on films immersed in ultrapure water. [40] At time t = 0 hrs (i.e. within 2 minutes after immersion in H₂O), the contact angle corresponding to the surface-modified polymer film was similar to the contact angle of the pTHF-U₄U (Figure 8.5). However, the water contact angles measured on dry films showed a value that was higher for the surface-modified (containing 24 mol% additive molecules) compared to the unmodified films (Figure 8.2). The results obtained using the captive air bubble method suggest that already within 2 min after immersion in H₂O, the surface of the modified polymer film rearranged to some extent and became more hydrophilic than in dry state. This points to a fast reorganization of the surface of the modified polymer film upon exposure to H₂O, in agreement with the previous measurements (Figure 8.4).
Figure 8.5 Snapshots of contact angle measurements, using the captive air bubble method, which show the contact angle of an air bubble with the surface-modified polymer film (containing 24 mol% additive molecules) (A) and the unmodified film (B) at time t = 0 hrs and after 18 hrs. Time t = 0 hrs was considered within 2 min after sample immersion in H2O, when the first measurement was recorded.

Moreover, after 18 hrs in contact with water, the surface of the polymer film containing additive molecules had become even more hydrophilic, as indicated by the increase of the contact angle with the air bubble from 126° ± 5° to 149° ± 4°. In the case of the unmodified pTHF-U4U film, the contact angle did not change significantly (Figure 8.5).

These results suggest that, in water, at least a part of the additive molecules at the surface of the films rearrange and expose the polar NH3+ groups to minimize the free energy of the water/film interface. This effect becomes more significant when the sample is exposed to water for a longer time.

Tapping Mode AFM investigation of surface-modified polymer films (containing 24 mol% of 2) that had been exposed to water for 48 hrs did not show, however, a significant difference in their morphology compared to the dry films (Figure 8.6). This suggests that even if a fast surface rearrangement was observed in the first minutes of exposure to water, the reorganization of the aggregates of 2 at the surface of the polymer film (exposed for 48 hrs to water) does not occur to such an extent that it has a significant influence on the surface morphology of the film. The surface of the modified polymer film immersed in water for 48 hrs was still completely coated with aggregates of the additive molecules (Figure 8.6 C).

Figure 8.6 Tapping Mode AFM phase images of pTHF-U4U films containing 24 mol% additive: (A) as prepared and (B-C) after 48 hrs exposure to water. Phase shift: 40° (A), 25° (B-C).
8.4 Annealing of the modified bisurea polymer films

With the aim of facilitating the reorganization of the additive molecules at the surface of the modified polymer films to expose the NH$_{3}^{+}$ groups, surface-modified films containing 24 mol % of 2 were annealed in water at 50 $^\circ$C. Contact angle measurements using the captive air bubble method revealed that the surface wettability increased significantly faster in the case of the films that were kept in water at 50 $^\circ$C compared to films that were immersed in water at r.t. Upon annealing for 3 ½ hrs in water, the surface-modified polymer film became too hydrophilic to allow attachment of the air bubble to its surface (Figure 8.7). The increased wettability of the film surface was tentatively assigned to an increased number of NH$_{3}^{+}$ groups of 2 present at the surface.

![Figure 8.7 Snapshots of contact angle measurements, using the captive air bubble method, on surface-modified polymer film, containing 24 mol% of 2, annealed in ultrapure water at 50 $^\circ$C. t represents the time of annealing.](image)

After annealing, the morphology of the surface-modified polymer film (containing 24 mol% additive molecule 2) significantly changed and ribbon-like aggregates could be identified on the surface. These ribbons were similar in height (∼ 6 nm) to the ribbon-like aggregates formed by 2 alone when cast from an aqueous suspension on a substrate (Figure 8.8). The aggregates formed by 2 in water have been thoroughly characterized within the laboratory. [32, 41] The ribbons observed on the surface of the modified polymer films (Figure 8.8 A) were shorter (a few hundred nm long) and less wide (35-40 nm) than the ribbons formed by 2 when cast from an aqueous dispersion, which were micrometers long and several tens to few hundred nanometers wide.

Based on AFM, IR and XRD investigations, a molecular model has been proposed [32] in which the ribbons formed by the bisurea surfactant 2 (Figure 8.8 B and Figure 8.10 E) were composed of an interdigitated bi-layer of molecules with the hydrogen bonds running in the length direction of the ribbon (Figure 8.10 F). In this model, the polar NH$_{3}^{+}$ groups of 2 are exposed at the surface of the aggregates.
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Figure 8.8 Tapping Mode AFM phase image of a pTHF-U₄U film containing 24 mol% additive molecule 2 after 20 hrs annealing in H₂O at 50 °C (A) and height image of ribbon-like aggregates formed by molecules of 2 when cast on glass from an aqueous suspension (B). Phase shift: 50°. Vertical scale of height image: 10 nm. (C, D) Height profiles of the ribbons indicated by the horizontal section lines in (A) and (B), respectively.

There were also some regions where a microphase separated fiber morphology similar to the one of the unmodified pTHF-U₄U film was visible underneath the ribbons (Figure 8.8 A). The observation of these incontinuities suggests that some of the additive molecules present at the surface of the polymer film were released into water during the annealing process. Nevertheless, approximately 80 % of the surface of the polymer film was still covered by the additive molecules, presenting their polar NH₃⁺ groups to the aqueous phase.

Figure 8.9 Experimentally determined atomic percentages of the various atoms identified in the upper 7-10 nm of the surface-modified polymer films (containing 24 mol% of 2), annealed in H₂O at 50 °C (20 hrs). Measured % is based on XPS spectra acquired at 0° with respect to the surface normal. For comparison, the experimentally determined atomic percentages of the non-annealed surface-modified polymer films and the atomic percentages calculated assuming that only molecules of 2 were present at the surface are also shown.

XPS investigation of the annealed surface-modified films (containing 24 mol% of 2) revealed that the upper 7-10 nm of the film had a chemical composition similar to the one of
the non-annealed films, confirming that the additive 2 was still present at the surface (Figure 8.9). The increase of the signal corresponding to C–O and/or C–N may be related to the presence of 1 in some regions at the surface of the annealed film, as evidenced also by AFM.

When thicker self-standing films were prepared by casting the polymer – surfactant 2 mixture into a mould, Tapping Mode AFM showed that the morphology of the as-prepared films containing 24 mol% of 2 was similar to the morphology of the thin films containing the same amount of additive molecules (Figure 8.10 A, B). However, after annealing these films in water at 50 °C for 20 hrs, ribbons with a high aspect ratio (few µm long and 150-200 nm wide) were observed at the surface (Figure 8.10 C, D). Moreover, the entire surface of the self-standing films was covered with ribbon-like aggregates, which were able to follow the topography of the rough polymer film underneath. Even more than in the case of the thin films, these ribbons looked similar to the ribbons formed by the bisurea surfactant 2 when cast from an aqueous suspension on a glass substrate (Figure 8.10 E).

![Figure 8.10 (A-D) Tapping Mode AFM phase images of pTHF-U4U films containing 24 mol% additive molecules as prepared (A-B) and after annealing in water at 50 °C for 20 hrs (C-D). (A) Thin film dropcast on glass; (B-D) thick (~ 2 mm) self-standing films. (D) is an enlargement of (C) (highlighted region). Phase shift: 25° (A, B, D) and 30° (C). (E) Phase image of ribbons formed by molecules of 2 when cast on a gold-coated glass substrate from an aqueous suspension. Being flexible, the ribbons can follow the topography of the underlying gold grains. (F) Schematic molecular picture proposed for the organization of molecules of 2 in the ribbons presented in (E).]
During annealing in water at 50 ºC, the mobility of the additive molecules increases, possibly allowing water molecules to penetrate inside the aggregates and disrupt their organization. [42] The amphiphilic molecules of 2 subsequently rearrange into the ribbon-like aggregates, which show a higher level of organization (Figure 8.10 F). The hydrophobic interactions are probably the driving force for the formation of these new aggregates. The process of formation of ribbon-like aggregates is more efficient in the case of the thick self-standing films (possibly due to the increased availability of 2 in the film), resulting in a surface completely covered with well-organized micrometer-long ribbons, which are believed to expose ordered arrays of NH$_3^+$ groups. The annealing temperature was set at 50 ºC in order to prevent the disintegration of the ribbon-like aggregates of 2, which is known to occur at 70 ºC. [41]

### 8.5 Crystallization experiments

In order to investigate the capability of these surface-modified polymer films to induce the controlled nucleation of CaCO$_3$, crystallization experiments were performed using a supersaturated Ca(HCO$_3$)$_2$ solution, following the Kitano procedure. [43] The thick, self-standing polymer films were allowed to float at the air-solution interface and the crystals obtained on the side of the film facing the bulk of the solution after 20 hrs were investigated by SEM. In control experiments performed on unmodified pTHF-U$_4$U films, only {10.4} rhombohedral calcite crystals were observed (Figure 8.11 A). [44]

![Figure 8.11 (A-B) SEM images of unmodified calcite crystals obtained on the pTHF-U$_4$U film (A) and on the as prepared surface-modified polymer film containing 24 mol% additive molecules (B). (C) SEM of vaterite crystals grown on the surface-modified polymer film annealed in H$_2$O at 50 ºC for 20 hrs prior to mineralization. Inset (C) and (D): higher magnification SEM images of vaterite crystals standing on their edge. Crystals were isolated after 20 hrs. (E) SEM of an edge-on vaterite crystal grown on the ribbon-like aggregates formed by 2 when cast from an aqueous suspension on glass. Crystals were isolated after 3.5 hrs.](image-url)
Also, on the surface-modified pTHF-U₄U films, containing 24 mol% additive molecules, used as prepared, randomly oriented {10.4} calcite was formed (Figure 8.11 B). However, on the surface-modified polymer films that had been annealed in water at 50 °C (20 hrs) prior to mineralization, predominantly vaterite – a metastable polymorph of CaCO₃ – was obtained. Moreover, all vaterite crystals showed the same disk-shape morphology and ~ 50-60 % of them were standing edge-on (Figure 8.11 C, D). This orientation suggests that these crystals are aligned with the [11.0] axis perpendicular to the polymer surface. The rest of the vaterite crystals were laying flat on the polymer films, being oriented with the (00.1) face parallel to the film surface. Control crystallization experiments on the ribbon-like aggregates formed by 2 cast on glass resulted also in the formation of vaterite crystals (Figure 8.11 E), together with calcite crystals. Also in this case, the crystals were disk-like and some of them were standing edge-on ([11.0] orientation).

The formation of vaterite on the ribbon-like aggregates of 2 (exposing NH₃⁺ groups) is in agreement with previous reports, which showed that positively charged monolayer templates such as octadecylamine induced oriented vaterite formation ([11.0] and [00.1] orientation). This has been attributed to the electrostatic binding of HCO₃⁻ ions orthogonal to the NH₃⁺ groups of the monolayer, which also provided orientational flexibility to accommodate particular crystal faces.

8.6 Conclusion

The results described in this chapter demonstrate that films of a bisurea thermoplastic elastomer, surface modified by addition of an amine functionalized bisurea molecule can be used as templates for CaCO₃ crystallization, resulting in the specific nucleation of the vaterite polymorph and control of the crystallographic orientation of the crystals formed.

Upon addition of more than 10 mol% of 2 to pTHF-U₄U, the surface of the polymer film became completely covered with aggregates of the additive molecules. In the as-prepared films, the alkyl chains of the additive molecules were exposed at the surface. However, annealing in water at 50 °C induced a reorganization of the molecules of 2 present at the surface of the surface-modified polymer film. Well-organized ribbon-like aggregates of 2, exposing an array of NH₃⁺ functional groups at the surface, were formed, not integrated in the film structure but persistent at the surface. While unmodified calcite crystals were obtained on the unmodified pTHF-U₄U films, the NH₃⁺ groups at the surface of the annealed surface-modified films induced the formation of [11.0] and [00.1] oriented vaterite crystals.

As it has been demonstrated that modified pTHF-U₄U polymers, containing guest molecules with complementary bisurea units, could be drawn into fibers of micrometer size (in diameter) by electrospinning, it should be possible, in principle, to fabricate 3D scaffolds consisting of a mesh of polymer fibers coated with an organized array of functional groups of the additive molecules 2 (Figure 8.12).
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Figure 8.12 Proposed model of a 3D porous scaffold consisting of a mesh of polymer fibers coated with an organized array of NH₃⁺ groups. The SEM image (right) corresponds to electrospun fibers of pTHF-U₄U containing 0.25 wt% of a dye with a complementary bisurea unit. [25]

After mineralization, these meshes of polymer fibers would be of particular interest as 3D scaffolds for bone cell culture. Using more biodegradable materials, such as polycaprolactone-based bisurea thermoplastic elastomers, [52] instead of the pTHF-U₄U polymer matrix, could lead to possible applications in bone tissue engineering.
8.7 Experimental section

**Materials.** The block copoly(ether)urea 1 was synthesized by R. A. Koevoets (Laboratory of Organic and Macromolecular Chemistry, Eindhoven University of Technology) according to a procedure previously described.\(^{[23]}\) The additive molecule 2 was synthesized by M. R. J. Vos and G. Etchebarria (Laboratory of Organic and Macromolecular Chemistry, Eindhoven University of Technology).\(^{[32]}\) See also Chapter 6 (Experimental Section).

**Polymer film preparation.** Glass substrates (microscopy cover glasses, diameter = 15 mm, Menzel-Glasser, Germany) were cleaned by ultrasonic treatment in acetone and dried with N\(_2\). Thin films of polymer 1, with and without additive molecule 2 mixed in, were prepared by dropcasting 5 mg/mL solutions in chloroform/methanol (8/2 v/v) on glass substrates. The samples were allowed to dry for one day. Different amounts of 2 were mixed in 1: 0.5 mol\% (0.02 wt\%), 2 mol\% (0.9 wt\%), 10 mol\% (4.3 wt\%), 24 mol\% (10.3 wt\%). The mol percentages were calculated with respect to the bisurea hard block of 1. Thick (~ 2 mm) self-standing films were prepared by casting 30 mg/mL solutions in chloroform/methanol in PTFE molds. The solvent was allowed to evaporate slowly by placing a beaker over the mould. After 20 hrs the films were dried in vacuum at r.t. for 5 hrs and peeled off the PTFE molds.

**Crystallization experiments.** See Chapter 6 (Experimental Section).

**Scanning Electron Microscopy (SEM).** See Chapter 5 (Experimental Section).

**Tapping Mode Atomic Force Microscopy (AFM).** AFM measurements were performed using a Multi Mode Scanning Probe Microscope (Nanoscope III) from Digital Instruments, Inc. (Santa Barbara, California). The samples were probed using NanoSensors PPP-NCH cantilevers (frequency: 300-330 kHz, force constant: 42 N/m).

**X-Ray Photoelectron Spectroscopy (XPS).** See Chapter 2 (Experimental Section).

**Contact angle measurements.** See Chapters 2 and 3 (Experimental Section)
8.8 References and notes

[6] E.g. poly(aspartic acid), pAsp, poly(glutamic acid), pGlu, poly(acrylic acid), PAA and aspartic acid rich proteins
[31] Eva Wisse (Laboratory of Organic and Macromolecular Chemistry, Eindhoven University of Technology) - unpublished results.

The surface-modified polymer films were washed in water prior to the measurements to remove any loose additive molecules that might have come out the polymer film into water and modify the contact angle values.
A control experiment performed on polytetrafluoroethylene (Teflon®) showed that the decrease of $\theta$ due to water evaporation was $\sim 2^\circ$ in 120 s.

An air bubble, being hydrophobic, spreads on a hydrophobic surface more than on a hydrophilic surface, resulting in a low contact angle on a hydrophobic surface and a high contact angle on a hydrophilic surface, respectively.

Matthijn R. J. Vos (Laboratory of Organic and Macromolecular Chemistry, Eindhoven University of Technology) - unpublished results

Previous DSC investigations have shown that pTHFU4U exhibited a glass transition temperature at -68 °C, corresponding to the amorphous pTHF block and a broad melting peak of the hard block with a maximum at 131 °C. The flow temperature of the polymer (i.e. the temperature at which the material loses its dimensional stability) was $T_{\text{flow}} = 140 °C$, as determined by optical microscopy (see references [17] and [23]). Therefore, a reorganization of the entire elastomer is not expected during the annealing of the surface-modified polymer films at 50 °C.


In the absence of polymer films, only unmodified calcite and some vaterite crystals were isolated from the crystallization solution.

In some cases, the films had fallen to the bottom of the crystallization dish. On those films, modified calcite crystals were observed, probably due to different mineralization kinetics at the bottom of the dish compared to the air/solution interface. The degassing rate of CO$_2$ from the supersaturated calcium bicarbonate solution is faster at the air/water interface than in the bulk of the solution.


It is also possible that some of the vaterite crystals that had nucleated with the [11.0] axis perpendicular to the polymer surface have fallen during sample handling and they appear laying flat on the polymer film in the SEM images.


These samples were placed at the bottom of the crystallization dish during the experiment, so the possibility that non-modified calcite crystals from solution had fallen on the ribbon-covered glass slides cannot be excluded.


Summary

The research described in this thesis applies concepts from Nature to create biomimetic structures, which can be used to study and elucidate processes and interactions in biological systems. More specifically block copolymer films are used to create patterns that can be employed to study the formation and organization of muscle tissue in vitro, whereas mineralized polymer patterns are used to monitor the interaction of bone cells with calcium carbonate (CaCO$_3$)-based biomaterials. In addition self-organizing supramolecular structures with a defined chemistry and organization are employed to investigate the mechanisms of CaCO$_3$ crystallization controlled by organic templates.

The first part of the research focuses on using patterned films of amphiphilic ABA block copolymers to control muscle cell attachment and alignment, as well as the patterned deposition of CaCO$_3$ films. Chapter 2 presents the selective adhesion/delamination behavior of POEGMA-based* copolymer films coated on hydrophobic and hydrophilic substrates. Upon exposure to water the films deposited on hydrophilic substrates undergo a fast rearrangement generating a hydrophilic surface before they delaminate from the substrate. In contrast, when deposited on a hydrophobic substrate, the surface rearrangement of the same copolymer films is less pronounced and they remain as coherent thin films on the substrate, despite the fact that on both types of substrates the films possess identical microphase separated surface morphologies. Based on XPS and AFM data it is proposed that this selective adhesion/delamination is a direct consequence of the self-organization of the block copolymers in the thin films. The selective delamination from hydrophilic regions of a substrate upon exposure to an aqueous solution is exploited in Chapter 3 to generate, in one step, patterned substrates of copolymer-coated gold lanes on glass. The cell-resistant OEG groups in the POEGMA block at the surface of the polymer lanes are used to induce the alignment of C2C12 mouse myoblasts on the uncovered glass lanes. The myoblasts are differentiated into 400-500 µm long, parallel oriented myotubes that show contraction upon electrical stimulation, thereby forming a promising 2D in vitro model for muscle. In Chapter 4, conducting PEDOT† substrates are photolithographically patterned using ABA polysilane-based block copolymers, PHEMA-PMPS-PHEMA‡ which do not show the selective delamination. The resulting substrates, consisting of cell-adhesive (PEDOT-PSS) and non-adhesive (PHEMA-PMPS-PHEMA) regions are successfully employed to control muscle cell attachment and alignment. Using these conducting polymer substrates the electrical stimulation of myotubes, which contract at the imposed frequency when alternating electric potential pulse were applied, is also demonstrated. In addition, the surface properties of the conducting polymer patterns can be controlled electrochemically, either by deposition of another conducting polymer or by applying an electric potential to change the oxidation state of the electroactive materials. In principle, substrates with locally tunable surface properties may find application in the production of engineered tissues consisting of multiple cell lineages derived from the same stem cell type. In Chapter 5, photolithographically patterned films of the PHEMA-PMPS-PHEMA block copolymers are used, in combination with the possibility to shape CaCO$_3$ in its amorphous state, to create patterns of CaCO$_3$ films. When the mineral is deposited onto a polymer film previously irradiated with UV-light through a mask,

* POEGMA – Poly[oligo(ethylene glycol) methyl ether methacrylate]
† PEDOT - Poly(3,4-ethylenedioxythiophene)
‡ PHEMA – Poly(hydroxyethyl methacrylate); PMPS – Poly(methylphenylsilane)
the amorphous CaCO$_3$ layer grown on the irradiated lanes can be selectively removed upon immersion of the film in ethanol. This results in the formation of a pattern of amorphous mineral film lanes, which upon standing become crystalline. Preliminary cell culture experiments show that rat bone marrow stromal cells are able to attach and differentiate into osteoblast-like (i.e. bone producing) and osteoclast-like (i.e. bone resorbing) cells on the crystalline CaCO$_3$ films. Therefore, the patterns of CaCO$_3$ films may serve as a 2D model system for CaCO$_3$ biomaterials with which the interaction of bone cells with the mineral can be studied.

The second part of the research concentrates on using self-organized systems based on supramolecular interactions as 2D templates for controlled CaCO$_3$ crystallization. In Chapter 6 and Chapter 7, self-organizing Langmuir monolayer systems are employed to study the relationship between the organization and flexibility of the template and the polymorph, morphology and orientation of CaCO$_3$ crystals nucleated underneath. The first system, presented in Chapter 6, is based on a water soluble (Leu-Glu)$_4$ octapeptide modified with a phospholipid tail in order to increase its amphiphilicity. This lipopeptide forms a β-sheet structure at the air-water interface thereby exposing an ordered array of carboxylic acid groups to the aqueous phase, which acts as a biomimetic mineralization template for the formation of a new crystal habit of calcite. For comparison the effect of the less adaptable N-acetylated octapeptide monolayer on the crystallization of CaCO$_3$ is also investigated. The results of the mineralization experiments demonstrate that the nucleation of different crystal faces can be achieved depending on the ability of the template to adapt to the structure of the inorganic phase. In Chapter 7, monolayers of bisurea-based amphiphiles, consisting of an alkyl chain, a bisurea unit and different amino acids as head groups are used. By varying the amino acid head group (i.e. from glycine to alanine to valine), it is possible to control the molecular packing density and the flexibility of the monolayer templates. These parameters significantly influence the ability of the monolayer template to induce oriented nucleation of calcite, as well as the crystallographic orientation of the resulting crystals. As a consequence, the most well-organized, rigid, glycine-based monolayer acts as a poor template, leading to mainly non-specific crystal nucleation. In the flexible valine-based monolayer, the molecules can rearrange to minimize the geometrical and orientational lattice mismatch with the nucleating crystal face. Thus the interfacial tension of the system is reduced and the nucleation of uniformly [10.0] oriented calcite crystals is promoted. In agreement with Chapter 6, these results confirm that the capability of a monolayer template to direct nucleation and growth of uniformly oriented crystals is related to its ability to adapt to the structure of the inorganic phase. Chapter 8 describes the use of films of a bisurea-based thermoplastic elastomer, surface-modified by addition of an amine functionalized bisurea molecule, as 2D templates for controlled CaCO$_3$ crystallization. The additive molecules form well-organized ribbon-like aggregates, exposing an array of NH$_3^+$ functional groups, not integrated in the polymer film structure but persistent at the surface. These aggregates induce the nucleation of the vaterite polymorph and a specific crystallographic orientation of the crystals. These surface-modified polymers offer in principle the possibility to fabricate 3D scaffolds consisting of a mesh of polymer fibers coated with an organized array of functional groups. After mineralization, these 3D scaffolds may be used for bone cell culture, with possible applications in bone tissue engineering.
Samenvatting

Het onderzoek beschreven in dit proefschrift gebruikt concepten uit de natuur om biomimetische structuren te creëren die gebruikt kunnen worden om processen en interacties in biologische systemen te bestuderen en te ontrafelen. Meer specifiek worden films van blokcopolymeren gebruikt om de vorming en organisatie van spierweefsel in vitro te bestuderen en worden gemineraliseerde polymere patronen gebruikt om de interactie van botcellen met calciumcarbonaat (CaCO₃) gebaseerde biomaterialen te onderzoeken. Verder worden zelforganiserende supramoleculaire structuren gebruikt om inzicht te verkrijgen in de mechanismen die actief zijn tijdens de kristallisatie van CaCO₃ onder invloed van organische dragermaterialen.

Het eerste deel van het onderzoek richt zich op het gebruik van patronen van dunne lagen van ABA blokcopolymeren die worden gebruikt om de hechting en uitlijning van spiercellen te controleren. Tevens richt het zich op de afzetting van patronen van CaCO₃. Hoofdstuk 2 beschrijft de selectieve hechting/onthechting van dunne lagen die bestaan uit POEGMA* copolymeren die zich bevinden op hydrofobe en hydrofiele substraten. In de aanwezigheid van water ondergaan de dunne lagen op de hydrofiele ondergrond een snelle reorganisatie waarbij een hydrofiel oppervlak wordt gevormd voordat de laag zich losmaakt van het substraat. Dit in tegenstelling tot lagen die zich op een hydrofoob substraat bevinden. De reorganisatie van het copolymeeroppervlak daarop is minder uitgesproken en de dunne lagen blijven aan het substraat gehecht, ondanks het feit dat op beide substraten de polymerfilms een identieke microfase-gescheiden oppervlaktetemorologie hebben. Op basis van XPS en AFM metingen wordt voorgesteld dat de selectieve hechting/onthechting een direct gevolg is van de zelforganisatie van de blokcopolymeren in de polymere films. De water-geïnduceerde selectieve delaminatie van deze polymeerlagen wordt in Hoofdstuk 3 gebruikt om in één stap patronen te maken van copolymeer-gecoate goud-banen op een ondergrond van glas. De cel-aftotende OEG groepen van de POEGMA blokken die zich aan het oppervlak van de polymere strepen bevinden worden gebruikt om de uitlijning van C2C12 myoblasten te induceren. De myoblasten differentiëren naar 400-500 µm lange, parallel georiënteerde myotubuli die sametrekken bij elektrische stimulatie, waardoor een veelbelovend 2D in vitro model van een spier is verkregen. In Hoofdstuk 4 worden op een fotolithografische wijze patronen gevormd van geleidende PEDOT† substraten door gebruik te maken van de polysilaan-houdende ABA blokcopolymeren, PHEMA-PMPS-PHEMA‡, die geen selectieve onthechting vertonen. De verkregen substraten, die bestaan uit cel-hechtende (PEDOT-PSS) en cel-aftotende (PHEMA-PMPS-PHEMA) gebieden, worden succesvol toegepast om de hechting en uitlijning van spiercellen te controleren. Bovendien wordt de elektrische stimulatie van myotubuli gedemonstreerd, die sametrekken met dezelfde frequentie als de elektrische wisselspanning die wordt opgelegd aan de geleidende polymere substraten. Ook blijkt het mogelijk om de oppervlakte-eigenschappen van de geleidende polymeerpatronen elektrochemisch te beïnvloeden, zowel door het laten neerslaan van elektro-actieve materialen als door er een elektrische spanning op te zetten waardoor de oxidatietoestand van de elektro-actieve materialen wordt veranderd. Deze substraten, waarbij

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Samenvatting

locaal de oppervlakte-eigenschappen kunnen worden aangepast, zijn veelbelovend voor het kunstmatig creëren van weefsels waarbij verschillende gedifferentieerde celtypen uit dezelfde stemcel zijn ontstaan. In Hoofdstuk 5 worden dunne lagen van PHEMA-PMPS-PHEMA blokcopolymeren gebruikt, waarin op een fotolithografische wijze patronen zijn gemaakt om hiermee patronen van CaCO₃ te creëren. Er wordt getoond dat het mineraal in zijn amorphe vorm op de polymere film kan worden afgezet. Wanneer deze film van te voren door een masker wordt beschoten met UV licht kunnen de amorfe CaCO₃ lagen selectief van de belichte gebieden worden verwijderd door het geheel in ethanol te dopen. Hierdoor ontstaat een patroon bestaande uit een dunne laag van CaCO₃. Inleidende experimenten met celculturen tonen aan dat rat beenmerg cellen kunnen hechten aan de CaCO₃ substraten en kunnen differentiëren naar osteoblasten (bot-vormend) en osteoclasten (bot-afbrekend). Hierdoor zijn de CaCO₃ patronen veelbelovend als 2D model systemen waarmee de interactie van botcellen met het mineraal kan worden bestudeerd.

Het tweede deel van dit proefschrift richt zich op het gebruik van zelforganiserende supramoleculaire systemen als 2D sjabloon voor gecontroleerde CaCO₃ kristallisatie. In Hoofdstuk 6 en Hoofdstuk 7 worden zelforganiserende Langmuir monolaag-systemen toegepast om de invloed van de organisatie en flexibiliteit van het sjabloon op de polymorfsselectie, de morfologie en de oriëntatie van CaCO₃ kristallen onder te onderzoeken. Het eerste systeem, dat in Hoofdstuk 6 wordt beschreven, bestaat uit een wateroplosbaar (Leu-Glu)₄ octapeptide dat gekoppeld is aan een fosfolipide-staart zodat de amfifiliteit wordt vergroot. Dit lipopeptide vormt een β-sheet structuur aan het lucht-water grensvlak waardoor een naar het water gerichte, geordende matrix van carbonzuur-groepen wordt gevormd, die blijkt te kunnen dienen als een biomimetisch mineralisatie-sjabloon voor de vorming van een nieuwe morfologie van calciet. Ter vergelijking is de kristallisatie van CaCO₃ op een N-geacetyleerde octapeptide monolaag ook bestudeerd. De resultaten van de mineralisatie-experimenten tonen aan dat nucleatie van verschillende kristalvlakken kan worden verkregen afhankelijk van het vermogen van de sjabloon om zich aan te passen aan de structuur van de anorganische fase. In Hoofdstuk 7 worden monolagen van amfifilieën gebruikt, die bestaan uit een alkylstaart, een bisurea-eenheid en een kopgroep die bestaat uit wisselende aminozuren. Door de aminozuur-kopgroep te variëren (door glycine, alanine of valine te gebruiken), is het mogelijk om controle te verkrijgen over de moleculaire dichtheid van de pakking en de flexibiliteit van de monolaag-sjablonen. Deze parameters blijken een significante invloed te hebben op het vermogen van het monolaag sjabloon om een georiënteerde nucleatie van calciet te induceren als wel op de kristallografische oriëntatie van de gevormde kristallen. De meest georganiseerde, dicht gepakte monolaag die glycine kopgroepen bevat, is de minst goede sjabloon, die vooral een niet-specifieke kristalnucleatie blijkt te geven. In het geval van de flexibele monolaag die valine-kopgroepen bevat kunnen de CaCO₃ moleculen zich herschikken om op die manier de geometrische en oriëntationele roosterfouten met het groeiende kristalvlak te minimaliseren. Hierdoor wordt de grensvlakspanning van het systeem gereduceerd en wordt de nucleatie van uitsluitend {10.0} georiënteerde calcietskristallen bevorderd. In overeenkomst met Hoofdstuk 6 bevestigen deze resultaten dat het vermogen van een monolaag-sjabloon om nucleatie en uniform georiënteerde kristalgroei te induceren afhangt van het vermogen om zich aan te passen aan de structuur van de anorganische fase. Hoofdstuk 8 beschrijft het gebruik van dunne lagen van een thermoplastisch elastomeer dat bisurea-groepen bevat als 2D sjabloon voor gecontroleerde CaCO₃ kristallisatie, waarbij de oppervlakte van de polymere laag is aangepast door de
toevoeging van een amine-gefunctionaliseerd bisurea-molecuul. De toegevoegde moleculen vormen goedgeorganiseerde lint-achtige aggregaten, die een matrix van functionele NH$_3^+$ groepen aan het oppervlak aanbieden. De toegevoegde moleculen zijn niet geïntegreerd in de polymere film, maar vormen een stabiel oppervlak van lintvormige aggregaten. Deze aggregaten stimuleren de specifieke nucleatie van vateriet en een specifieke kristallografische oriëntatie van de kristallen. Deze polymere structuren met een gemo dificeerd oppervlak, bieden de mogelijkheid om 3D dragermaterialen te maken die bestaan uit een netwerk van polymervezels die gecoat zijn met een georganiseerde matrix van functionele groepen. Na mineralisatie kunnen deze 3D dragermaterialen mogelijk worden gebruikt om botcellen te kweken, hetgeen toepassingen biedt bij het kunstmatig produceren van weefsels.
CURRICULUM VITAE

Daniela Popescu was born in Bucharest (Romania) on February 7th, 1978. She graduated from “Grigore Moisil” High School (Bucharest) in 1996. In the same year she started studying Chemical Engineering at the “Politechnica” University of Bucharest. She graduated from the university in September 2001. She completed her undergraduate research project in Organic Chemistry under the supervision of prof. dr. S. Z. Zard at Ecole Polytechnique (France). In October 2001 she started as a PhD student in the Macromolecular and Organic Chemistry Group at the Eindhoven University of Technology under the supervision of prof. dr. E. W. Meijer and dr. N. A. J. M. Sommerdijk. The most important results of her PhD research are described in this thesis. As of December 2005, she works at Océ-Technologies B.V. in Venlo (the Netherlands).
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Daniela