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# Biomimetic Polymers for Chiral Resolution and Antifreeze Applications

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## 1. Introduction

Nature and natural processes have fascinated and inspired scientists for many years, mainly in order to develop new materials with enhanced properties based on knowledge stored in nature. Biological systems synthesize and tailor biopolymers for a specific purpose, in that way dictating their precise and desired activity. For example, enzymes, receptors, antibodies, structural proteins, DNA and RNA are all biopolymers constructed from a limited number of building blocks, with each of these biopolymers having its own specific action. Therefore, mimicking natural biopolymers encompasses many potential applications in medicine;<sup>1-2</sup> tissue engineering,<sup>3-8</sup> and drug delivery.<sup>9-14</sup> Among the variety of biomimetic research areas, the interactions of biopolymers with solids and crystals have attracted a great deal of attention in recent years.<sup>15</sup> Biomineralization<sup>16-18</sup> is the most prominent process in biological systems that incorporates biopolymers in the synthesis of a solid phase. Biomineralization can be generally defined as the production of biominerals namely, inorganic or organic crystals by living organisms. Examples for biominerals are calcium carbonate,<sup>16,19-20</sup> hydroxylapatite,<sup>21-23</sup> silicate, and iron oxide. Living systems often merge water soluble soft templates, mostly biopolymers, in the crystal growth process of biominerals and therefore the resultant biominerals are an inorganic / organic hybrid composite with hierarchical structures and unique morphology. This process is largely spontaneous and is controlled by a self-organization route. In nature, the shape and properties of a crystal are determined not only by the use of soft templates in the crystallization process, but also an insoluble template on which crystallization occurs, provides a route to control crystal shape and properties.<sup>24-25</sup>

This rather easy approach that results in complex structures and unique material properties inspired scientists in their search for new functional materials. The production of solid materials with remarkable functionalities usually requires the ability to control the structure, shape, size and orientation of crystals. The control of these properties through the use of the bio-inspired approach encompasses a great potential for many fields. Therefore, the use of synthetic soluble hydrophilic polymers and polyelectrolytes is advantageous for achieving control over crystallization reactions.<sup>26-33</sup> The synthesis of bio-inspired soluble polymers for use as a soft template opens up the possibility to design polymeric model systems for the

study of crystallization processes, especially in biomineralization. The properties of the bio-inspired synthetic polymers can be pre-designed by determine the monomers sequence, resulting in the polymeric secondary structure. The feasibility to tailor polymeric properties motivated scientists to control other polymeric characteristics such as stereochemistry and chirality, mainly in order to achieve chiral recognition, and as a result, chiral separation upon crystallization.<sup>34</sup>

Copolymers, mainly double hydrophilic block copolymers (DHBCs), are defiantly playing an important role in mimicking natural polymers.<sup>35-48</sup> Copolymers or heteropolymers are polymers composed of two different monomers. The polymeric chain can have alternate ordered monomers known as alternated polymers, or two different polymeric blocks attached together, namely, a block copolymer. Block copolymers with amphiphilic properties are similar to some extent to low molecular weight surfactants.<sup>49-50</sup> These copolymers consist of hydrophobic and hydrophilic blocks. On the other hand, double hydrophilic block copolymers (DHBCs) have two polymeric blocks which are hydrophilic. One block is synthetically designed to have a strong specific interaction with a crystalline phase. The second block, mainly PEI (polyethyleneimine) or PEG (polyethyleneglycol), is largely responsible for the solubility and stabilization of the entire polymer in the aqueous medium. DHBCs are bio-inspired, and thus consist of blocks composed of natural building blocks such as amino acids (basic or acidic). The sequence and the number of amino acids can be controlled, and thus DHBCs have proven to be excellent model systems for the biomineralization process. DHBCs were also used for other applications such as the stabilization of nano-particles on metal species, semi-conductive materials, and as morphological modifiers.<sup>47,51-56</sup> The amino-acid moieties allow a fine tuning of other polymer properties, such as charge, and as a result, relative solubility by changing the pH of the solution.<sup>57</sup> Another important property is the possibility to control the chirality of the amino-acid moiety. The chirality of the DHBC moiety can be controlled at two different levels, the intrinsic chirality of monomers and the chirality of a secondary structure of the polymer namely, the transformation  $\alpha$ -helix and random coil by change of pH<sup>58</sup> and temperature.

This review will mainly focus on bio-inspired chiral polymers for application of chiral recognition and chiral separation. Therefore, we will first present a short introduction to chirality with the emphasis on chirality at the solid state. We will then review the latest advances in chiral recognition and chiral separation by biomimetic soluble and insoluble chiral polymers. The chapter will also describe the use of short peptides and polysaccharides for the inhibition of water crystallization for antifreeze applications.

## 2. Chirality<sup>59</sup>

The term "chiral" (from the Greek for 'hand') was first introduced into science by Lord Kelvin (William Thomson).

"I call any geometrical figure, or group of points, chiral, and say that it has chirality, if its image in a plane mirror, ideally realized, cannot be brought to coincide with itself." Lord Kelvin, 1884.<sup>60</sup>

The most significant cornerstone in the discovery of chirality was made by Louis Pasteur in 1848. Pasteur, by a simple crystallization of tartaric acid (TA) salts ( $\text{Na}_2\text{C}_4\text{H}_4\text{O}_6$ ), discovered the correlation between the dissymmetry in crystals and molecules. Pasteur defined Enantiomers as a pair of molecules that are related to each other as an object to its mirror

image. These molecules are non-superimposable and therefore are chiral. From this observation Pasteur laid the foundation for a new scientific field - Stereochemistry.

“Life is dominated by dissymmetrical actions. I can foresee that all living species are primordially, in their structure, in their external forms, functions of cosmic dissymmetry.”

Louis Pasteur, 1848.<sup>61</sup>

Historically, the origins of chirality and the discovery of plane-polarized light are related. In 1812, the French scientist, Biot,<sup>62</sup> observed that a quartz plate rotates the plane of polarized light some of the quartz crystal turned the plane polarized light to the right and some to the left. This constitutes the phenomenon of optical rotation. Hauy,<sup>63</sup> attributed the rotation of plane-polarized light to the fact that quartz crystals exhibit the phenomenon of hemihedrism. Hemihedrism implies that certain crystal facets are disposed to produce non-superimposable species [Figure 1.1] that are related as an object to its mirror image. Such mirror image crystals are called enantiomorphous (from the Greek for opposite, "enantios"). It was left to Louis Pasteur to extend this correlation from the world of crystals to the world of molecules.<sup>64-65</sup> Pasteur succeeded in separating two enantiomorphous crystals of ammonium sodium tartrate salts from a racemic mixture. Pasteur re-dissolved the enantiomorphous crystals separately and found that the solutions also rotate plane polarized light. This observation led Pasteur to understand the analog between crystals and molecules. In both cases, the power to rotate polarized light was caused by dissymmetry, i.e., the non-identity of a crystal or a molecule and its mirror images. The two molecules are thus “enantiomorphous” at the molecular level, namely enantiomers.

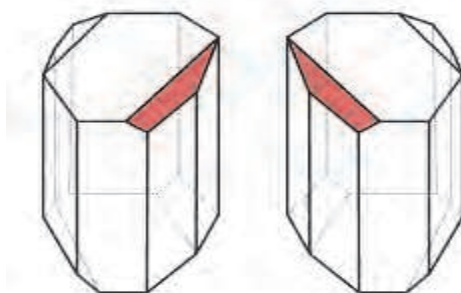


Fig. 1.1. The hemihedral faces of ammonium sodium tartrate.

Enantiomers have identical chemical and physical scalar properties. The magnitude and sign of these properties are invariant upon reflection. For instance, enantiomers exhibit an identical melting point, solubility, density, refraction index, IR, Raman, UV, NMR spectra, and an X-ray diffraction pattern. However, enantiomers differ in properties or manipulations that change the sign, but not the magnitude, upon reflection, e.g., optical rotation and circular dichroism (CD). Diastereomers are stereoisomers that are not related as objects and its mirror image, and often contain two or more chiral centers, chiral axes, or a combination of the two properties. In contrast to enantiomers, diastereomers differ in most (if not all) physical, and chemical properties. Pairs of enantiomers are usually referred as left- and right-handed. However, there are several common nomenclatures which indicate their exact configuration:

- *levo* (-) and *dextro* (+), which refer to the direction of the rotation of plane-polarized light. This notation does not refer to the absolute configuration of each of the enantiomers.

- D and L - adopt the same configuration as D- and L- glyceraldehyde
- R and S - defined by the Cahn-Ingold-Prelog rules<sup>66</sup>

### 2.1 Importance of chirality in the living system

Amino acids, the building blocks of proteins and enzymes, are 'left-handed', while all the sugar in DNA and RNA are 'right-handed'. Biological polymers must be homochiral in order to permit life as we know it.<sup>67</sup> Racemic polypeptides, composed of both left- and right-handed amino acids, could not form the specific shape required for enzymes. Wrong handed amino acids disrupt the stabilization of  $\alpha$ -helix in proteins. In addition, DNA could not be stabilized in an  $\alpha$ -helix, even if a single wrong-handed building block was present.<sup>68</sup> Biopolymers such as enzymes and receptors are folded into a specific structure that gives a well-defined cavity that can only bind a molecule with an exact orientation. Thus, proteins react differently with two enantiomers. While one enantiomer perfectly fits the specific cavity provided by the bio-polymer, its mirror image enantiomer will not, or only partially, bind to the same cavity. A tragic reminder of the importance of enantioselection in nature is the case of thalidomide. In the early 1960s, thalidomide was prescribed to pregnant women suffering from morning sickness. However, while the left-handed form is a powerful tranquillizer, the right-handed form disrupts the critical pathways required for fetus growth, resulting in severe birth defects. When the cause for the birth defects was discovered as arising from the use of racemic thalidomide, the drug was banned. Overall, during the last decades, because of scientific and economic reasons, there has been an increase in chirality research, with the pharmaceutical industry being the main contributor and driving force.

## 3. Chiral polymers<sup>69-70</sup>

Chiral synthetic polymers interest scientists for many reasons, but largely for application in chiral resolution and chiral recognition. As already mentioned most naturally occurring polymers are optically active and exhibit molecular recognition abilities owing to their specific chiral structure. The chirality of natural polymers can be expressed in different levels; in their primary structure, a chain of chiral building blocks, secondary structure,  $\alpha$ -helix and tertiary structure, the incorporation of several helical and  $\beta$ -sheets polymers to give a superstructure with a determined chirality, such as enzymes. Scientists attempted to mimic these natural chiral polymeric structures and to meet the challenges in developing diverse synthetic routes to construct functional chiral polymeric systems. Synthetic chiral polymers are obtained by different synthetic approaches: (1) Polymerization of chiral monomers. (2) Chiral post modification of chiral or non-chiral polymers and (3) Polymerization of both chiral and non chiral monomers to form helical polymers with either left or right handed configurations. In the next paragraphs we will elaborate on the synthetic routes for obtaining chiral polymers. The overall polymerization of chiral polymers can be classified into the following three major categories.

### 3.1 Asymmetric synthesis polymerization<sup>71</sup>

In asymmetric polymerization, an optically-inactive prochiral monomer or a prochiral monomer with an optically-active auxiliary is polymerized to give a polymer with chiral configuration at the main polymer chain.<sup>72-73</sup> In the polymerization reaction, the growing species attacks the monomer enantioselectively, on one enantioface, and thus, chiral centers

of one-handedness are obtained. This type of polymerization has been reported for various types of olefinic compounds, although the degree of asymmetric induction is unclear in most cases. The polymers of dienes and cyclic olefins can be optically active when the chiral centers in the main polymer chain have, preferentially, one of the two possible configurations (*R* or *S*), since the chiral centers can be true asymmetric centers.

### 3.2 Helix-sense selective polymerization<sup>69,74-75</sup>

In helix selective polymerization, optically active polymers whose chirality is based on a helical conformation with an excess of one single screw-sense polymers are produced. Because the right- and left-handed helices are mirror images, if one of the two is preferentially synthesized, the polymer can be optically active.<sup>76-77</sup> Although many stereoregular polymers have a helical conformation in the solid state, most of them cannot maintain a helical conformation in solution because the dynamics of the polymer chain are extremely fast in solution, except for some polymers having an optically-active side group.<sup>78</sup> Therefore, isotactic polystyrene<sup>79-80</sup> and polypropylene<sup>81</sup> prepared with an optically-active catalyst do not show optical activity due to a helical conformation. However, it is possible to obtain optically active polymers whose chirality is based on the helical structure of polymer when the rigidity of the polymer backbone or the sterical repulsion of the side groups prevents random conformation. The screw-sense of the polymer helix is produced by the chirality of the initiator (catalyst) or of the monomers. For example, in the asymmetric synthesis of helical bulky polymers such as polymethacrylates, the helical conformation is formed under kinetic rather than thermodynamic control, meaning that when the monomer inserts into the chain end it adopts its helical conformation. Once formed, these conformations are locked in by the high helix inversion barriers of these polymers. The first helix-sense selective polymerization was achieved from the monomer triphenylmethyl methacrylate, leading to a nearly 100% one-handed helical polymer during polymerization with a chiral anionic initiator. The one-handed helical polymethacrylates show excellent chiral recognition ability when used as a chiral stationary phase for high-performance liquid chromatography (HPLC).<sup>76</sup>

### 3.3 Enantiomer-selective polymerization<sup>82-85</sup>

In enantiomer-selective polymerization, one antipode of racemic chiral monomers is preferentially polymerized to give optically-active polymers. In stereoselective polymerization, a racemic mixture of the chiral monomers is polymerized to give a mixture of polymers preferentially consisting of one antipode with one enantiomer. In this process, the kinetic optical resolution of the racemic monomer is obtained. A well-known example of the enantiomer selective polymerization process is the ring opening of  $\alpha$ -amino acid *N*-carboxy anhydrides.

## 4. Resolution of enantiomers

Chiral resolution is the process of the separation of racemates into their enantiomers. Overall resolution methods may involve physical, chemical and biological processes. The resolution of enantiomers has interested scientists ever since the discovery of chirality, and up to the present. More than 100 years ago, Pasteur tried to induce a preference for right or left handed molecules by performing reactions in a centrifuge, and even by rotating growing plants to change the handedness of their natural products, but without pronounced success.

Scientists are highly motivated to resolve enantiomers not only due to extremely fascinating fundamental aspects, but also due to practical needs for determining the biological activity of each enantiomer for drug development. The resolution of racemates, in general, requires the presence of a chiral environment. For this purpose, chiral catalyst auxiliaries or chiral selectors are necessary. The chiral environment induces the formation of two diastereomeric species, which imply an energetic difference between them, thereby allowing for their chiral discrimination.

#### 4.1 Molecular-imprinted chiral polymers (MICP)<sup>86</sup>

Molecular imprinting, can be defined as the sculpted of specific cavities, binding sites, in a polymeric matrix.<sup>87</sup> This process involves the use of a template molecule which can be covalently attached to, or specifically interacts via weak forces with monomeric functional groups, thus promoting the formation of a unique cavity upon polymerization. Template removal is consequently accomplished by either chemical cleavage or simple extraction in a proper solvent. This liberates the corresponding functional groups located within polymer-embedded cavities. The size, shape, and functional-group arrangement of these cavities is complementary to the template molecule, and hence can act as template-specific chiral binding sites.<sup>88</sup> The first illustration of the preparation of Molecular Imprinted Polymers (MIPs) by the covalent approach was the pioneer research presented by Wulff [Figure. 4.1].<sup>89-90</sup>

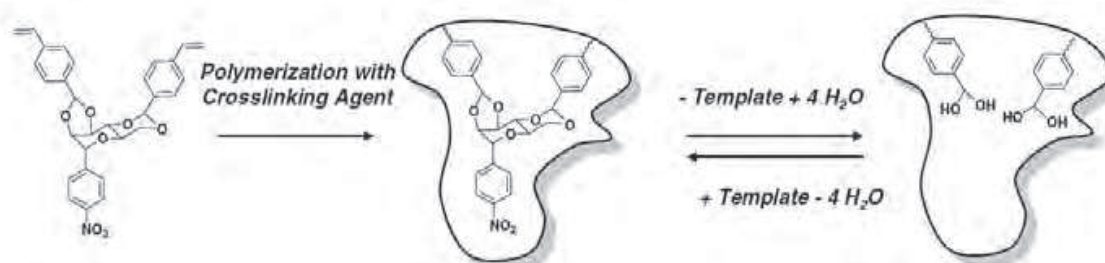


Fig. 4.1. Representation of Covalent Imprinting<sup>89</sup>.

The non-covalent imprinting approach was developed by Mosbach<sup>91</sup> in order to avoid the synthetic efforts associated with the formation of monomeric template molecules. Among its many applications, MIPs are a natural choice for the preparation of a solid phase with chiral cavities for the selective adsorption of enantiomers.<sup>92-93</sup> The most pronounced application is the design of new Chiral Stationary Phases (CSPs), mainly for High Performance Liquid Chromatographic (HPLC) applications. Most of the CSPs are non target-specific, and thus the use of molecular imprinting technologies provides the ability to tailor the solid phase for a desired enantiomer resolution. As a result, the molecular-imprinting approach has been extensively used to produce target-specific CSPs for a broad range of chiral compounds,<sup>94-96</sup> e.g., amino acid derivatives,<sup>97</sup> peptides,<sup>98</sup> natural compounds, and a variety of drugs.<sup>94</sup> In general, MIP-type CSPs have excellent chiral recognition properties for the template chiral species, which are pronounced in high enantioselectivity, high substrate-specificity, and predictable order of elution, with the enantiomers employed as templates being the more strongly retained species. A particularly attractive feature of MIP-type CSPs is their capability to discriminate, not only between enantiomers, but also between structurally closely-related stereoisomers. For example, a Poly(methyl methacrylate-co-ethylene

dimethacrylate) [poly(MAA-co-EDMA)], CSP imprinted with N-acryl-L-Phenylalanine-L-Tryptophan-OMe could successfully distinguish the template from the corresponding DD, DL, and LD isomers with remarkable selectivity factors.<sup>98</sup> MIP-type CSPs were synthesized as polymeric supports, silica beads, and silica films as monolithic supports. Much effort has been made in the synthetic improvement of MIP-type CSPs in order to maximize their potentials. A crucial limitation in the synthesis of MIP-type CSP particles for chromatographic purposes was the inability to control particle's morphology, roughness, pore size and size distribution. Therefore, the synthesis of silica and polymeric particles is continually being improved. Hosoya and Haginaka<sup>99</sup> illustrated the preparation of enantioselective uniform MIP particles in suspension polymerization of a two-step swelling process. These particles were templated with diamionaphthalene or a chiral amide derived from (S)- $\alpha$ -methylbenzylamine and showed chiral recognition for a variety of chiral pharmaceutical compounds, such as profens, calcium-channel blockers and antihistamines. Mosbach reported on water free suspension polymerization, an alternative path to the conventional suspension polymerization procedures for the preparation of MIPs in a particle format.<sup>100-101</sup> Mosbach used perfluoroalkane solvents to form a stable emulsion in a progenic solvent. The polymerization process carried out in the perfluoroalkane droplets results in relatively narrow- size distribution of MIP particles. Recently, Kempe illustrated a simplified synthetic approach for suspension polymerization. Kempe formed a suspension in mineral oil that served as a highly efficient dispersion medium in which polymerization took place. By this simplified polymerization approach, (S)-propranolol-imprinted spherical MIP particles were synthesized and showed good separation properties. In a series of papers, Sellergren described the development of MIP films by grafting methods.<sup>102-107</sup> His group used silica particles bearing surface-immobilized, free radical azo-initiator species to favor grafting polymerization on the particle's surface over polymer growth in solution. In his research, L-phenylalanine anilide was used as a template in the polymerization of MAA and EDMA functionalized monomers.

#### 4.2 Template-based imprinting approach

Although molecular imprinting methods allow the preparation of materials with high affinity and selectivity for given target molecules, some of their limitations prevent their use in real applications. Such limitations are non-specific binding; slow mass transfer, low sample-load capacity, and poor recognition in aqueous systems. Thus, a new approach for templating was introduced by Brinker and co-workers. Brinker followed in the footsteps of Pauling and Campbell, who were the first to mimic an antibody by the patterning of an antigen. In addition, Dickey which introduced his templating approach in which selective silica was prepared using a sol-gel process. The templating process of a silica network was formed in the presence of the target compound to be adsorbed. Brinker stated that "a central structure about which a network forms in such a way that removal of the template creates a cavity with morphological and/or stereochemical features related to those of the template". It is clear from this definition that template-based approaches can result in the formation of chiral nanoporous structures. For example, Alvaro *et al.*<sup>108</sup> used chiral binaphthyl precursors with trialkoxysilane TEOS for the preparation of optically-active porous material that linearly rotates polarized light. Corma *et al.*<sup>109</sup> used chiral trialkoxysilane grafting onto mesoporous silica materials, forming a whole range of chiral catalysts. In addition, there were reports on antibody-based bionanotube membranes for enantiomeric drug separation.<sup>110</sup> Chiral imprinting of sol-gel thin films exhibiting enantioselectivity has been



developed by David Avnir's group. In a series of articles, Avnir *et al.*<sup>111-112</sup> showed that template molecules such as propranolol, 2,2,2-trifluoro-1-(9-anthryl) ethanol, DOPA, and tyrosine can be used to prepare a chiral imprint sol-gel matrix. The shape of the chiral matrix is maintained when the template molecule is extracted. Therefore, the porous materials formed are enantiopure; i.e., the cavity left inside the sol-gel films can discriminate between optical enantiomers.

Recently, we demonstrated<sup>113</sup> a new approach for chiral imprinting onto a solid support based on the use of chiral block copolymers. In this method, silica is templated by the chiral DHBCs of a poly (ethylene oxide) block and a chiral block of amino acids such as D-phenylalanine [PEO-*b*-D-Phe]. In general, the synthesis of the chiral DHBCs is based on a simple ring opening polymerization of protected amino acid *N*-carboxyanhydrides (NCAs). This process results in the formation of chiral block copolymers based on PEO<sub>*n*</sub>-*b*-D- or L-amino acids. The obtained chiral DHBCs were then suspended in an acidic solution and the amino acid block found to have a helical structure, It's helically was determined by CD measurements. The ability of chiral DHBCs to aggregate into well-defined micelles at the reaction conditions was crucial to the formation of a well-defined template. The chiral DHBCs aggregation behaviour was achieved by the change in the DHBC concentration in the reaction solution. As a final step a silica processor was added to the reaction solutions containing the chiral DHBC and the sol-gel process was accomplished. As a subsequent step the chiral template was removed by solvent extraction, leaving chiral voids. Therefore, a block co-polymer with a D-phenylalanine block composed of ten repeating units in average was synthesized. The copolymers exhibit spherical micelles with a 10 nm diameter at the pH (pH=2) reaction. The sol-gel condensation of a silica processor, TEOS, was used to form silica in the presence of the chiral polymers in their Critical Micelle Concentration (CMC) to give the chiral imprinted mesoporous silica. Consequently, after the chiral DHBC template was removed, chiral hexagonal cavities of 5 nm diameter and a high surface area (700 gr/m<sup>2</sup>) were obtained. [Figure 4.2]

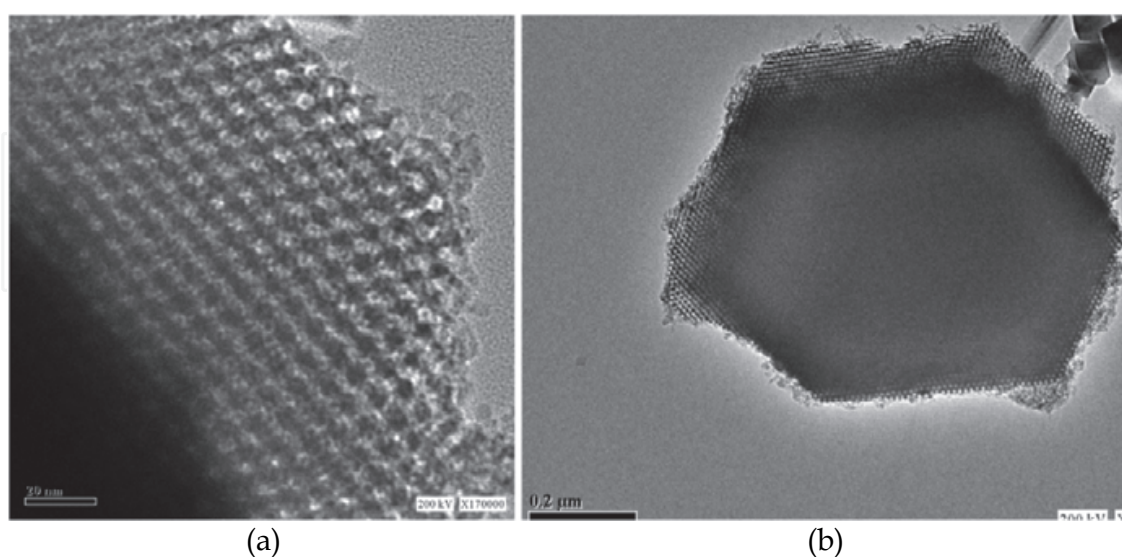


Fig. 4.2. TEM images: (a) high magnification (b) low magnification of chiral silica made from PEO<sub>113</sub>-*b*-D-Phe<sub>10</sub> after chiral copolymer extraction. The light area corresponds to the pores, while the dark area corresponds to the walls.

The chiral recognition ability of the obtained silica was examined by the selective adsorption of enantiomers from racemic solutions of D, L-valine. In these experiments, the chiral silica showed chiral recognition toward D-valine enantiomers, compatible to the chirality imprinted on the silica walls. The chiral recognition reached its maximum value after 16 hours and a chiral selectivity factor of 2.34 was calculated. Following our report on chiral imprinting of silica by chiral DHBCs, Paik *et al* <sup>114-115</sup> described the synthesis of chiral silica with chiral DHBCs in a series of papers. In their first paper, the authors described the synthesis of chiral mesoporous silica (CMS) spheres. The chiral mesoporous silica particles were prepared with the use of a chiral DHBC template of poly(ethylene oxide) block with a D/L-glutamic acid, [PEO<sub>113</sub>-*b*-(GluA)<sub>10</sub>] block [Figure 4.3]. The chiral template was extracted from the CMSs, leaving a chiral print on the silica walls. These particles exhibit a 2-3 nm pore size and a high surface area of 614 m<sup>2</sup>/g [Figure 4.3], and also show enantioselectivity towards valine enantiomers, corresponding to the chirality imprinted on the silica support (selectivity factor of 5.22 was calculated). In a sequential paper, Paik *et al* reported on the preparation of CMSs templated with DHBC of a poly(ethylene oxide)block with a D/L-aspartic acid block, [PEO<sub>45</sub>-*b*-(D/L-AspA)<sub>10</sub>]. These particles were subjected to D, L-valine and D, L-alanine solutions, and show high affinity toward one enantiomer corresponding to the chirality imprinted on the silica support. The selectivity factor was found to be remarkably high, 7.52.

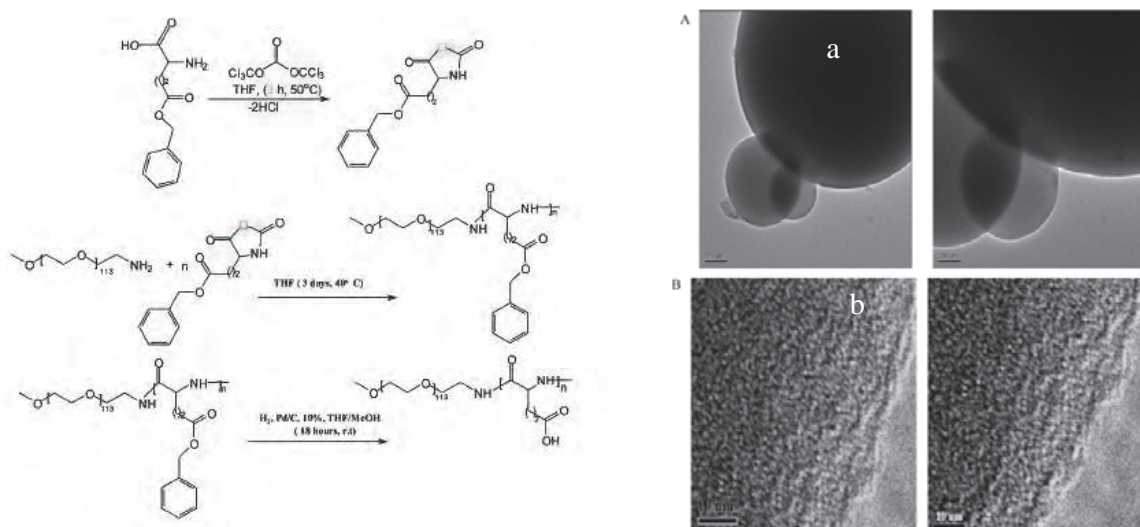
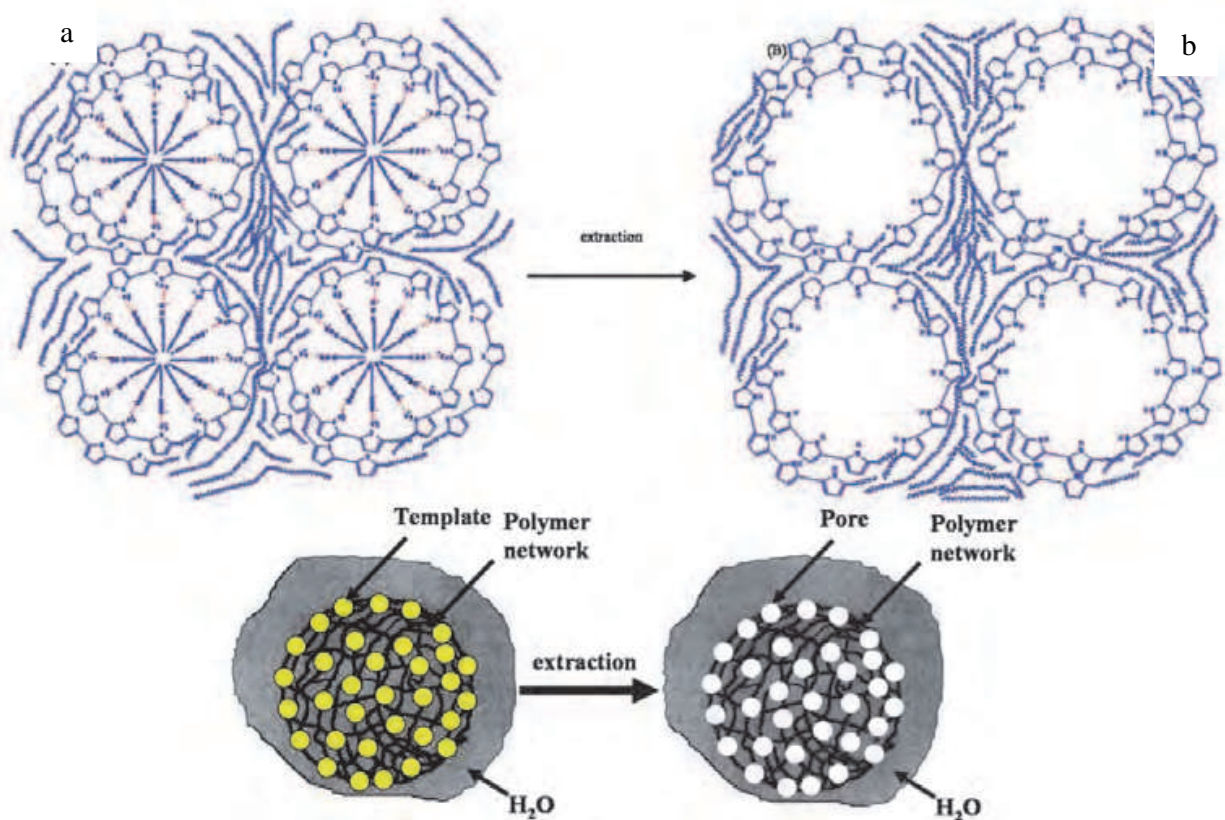


Fig. 4.3. Synthesis of the Block Copolymer of Poly(ethylene oxide)-L-glutamic Acid [PEO<sub>113</sub>-*b*-(L-GluA)<sub>n</sub>], where  $n = 10$ . HRTEM images: (a) low magnification (b) high magnification of CMS spheres (Ex-SiO<sub>2</sub>) synthesized by templating with PEG<sub>113</sub>-*b*-(L-GluA)<sub>10</sub>.

In a further paper, Paik *et al.*<sup>116</sup> demonstrated the feasibility of templating nanopolymeric particles by chiral DHBCs. In their report, chiral-mesoporous-polypyrrole (CMPPy) nanoparticles were synthesized by use of two DHBC templates based on poly(ethylene oxide) block with a L/D- glutamic acid block, [PEO<sub>113</sub>-*b*-(L/D GluA)<sub>10</sub>] and poly(ethylene oxide)block with a L/D phenylalanine block, [PEO<sub>113</sub>-*b*-(L-/D-Phe)<sub>10</sub>]. The synthesis of mesoporous polypyrrole nanoparticles was previously described by Fan *et al.* <sup>117</sup> The synthesis of chiral polypyrrole was carried out through a minor modification of Fan's polymerization procedure. In general, pyrrole monomers were added to a chiral DHBC

micelle aqueous solution. The pH of the reaction solution was adjusted to pH=2, and the polymerization of pyrrole monomers initiated in the presence of ammonium persulfate, the polymerization ended with the precipitation of a black nanoparticle powder. [Scheme 1] Consequently, the chiral DHBCs were removed by solvent extraction, leaving chiral mesoporous voids. The obtained chiral mesoporous polypyrrole had an average size of 300 nm with a pore size of 3.5 nm. The use of DHBCs for imprinting chirality onto a polymeric support was proven to be effective for chiral discrimination by adsorption. The CMPPy shown chiral discrimination of racemic mixtures valine and alanine enantiomers with the same chirality imprinted on the polymeric support. The maximum value of enantiomeric excess (e.e) observed in adsorption measurements was 54%, and a chiral selectivity factor of 5.05 was calculated for CMPPy. The authors also compared the chiral resolution power of CMPPy before and after extraction of the chiral polymers. Before solvent extraction, CMPPy shows a very low surface area and low chiral resolution capability.



Scheme 1. (a) Templating of chiral block copolymers (CBCs) for synthesizing the CMPPy, and electrostatic interaction between negatively charged carboxylic groups of CBC molecules and protonated imine nitrogens of polypyrrole (PPy), (b) after extraction of CBCs from the mesopores.

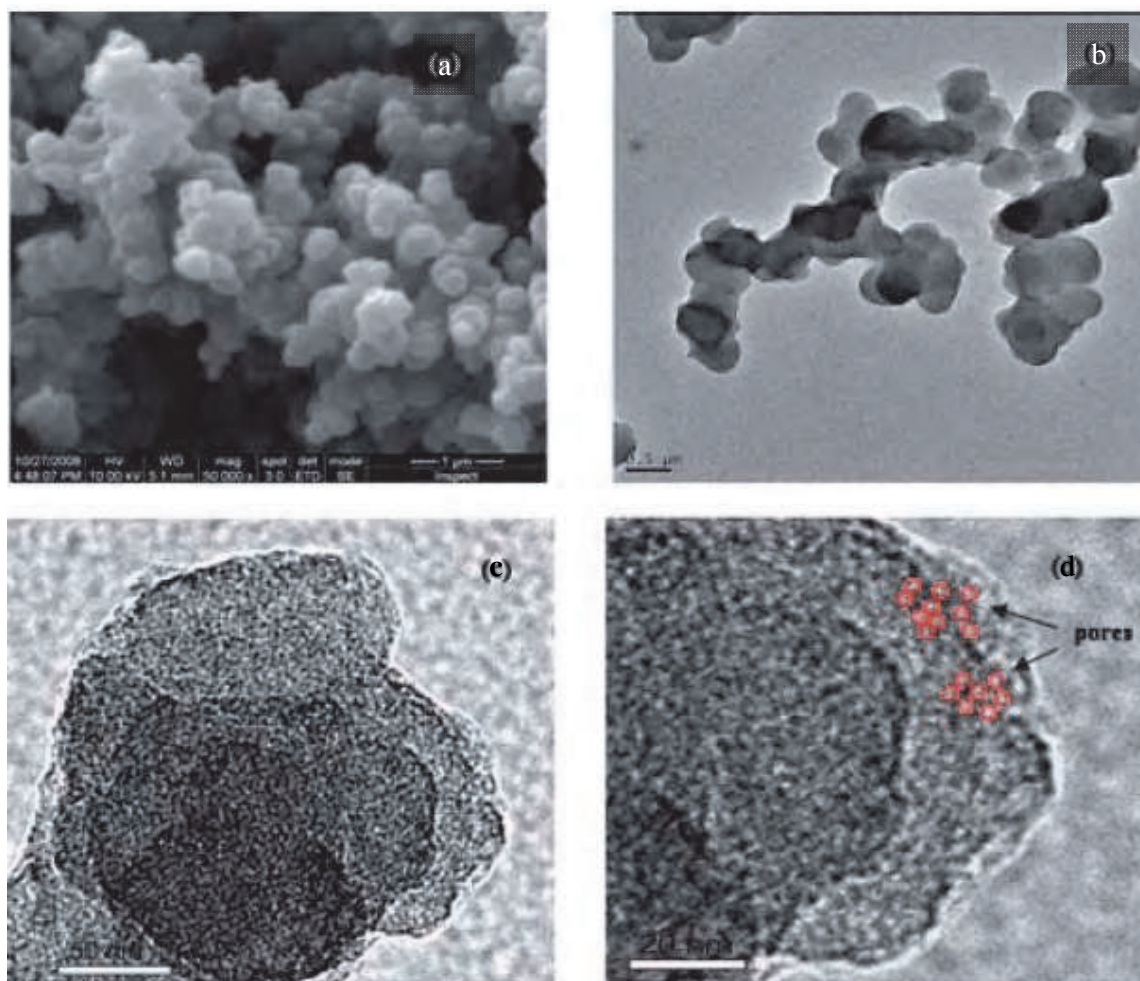


Fig. 4.3. (a) SEM (b) TEM (scale 50 nm) and (c, d) HRTEM images [scale 50 nm for (c) and 20 nm for (d)] of the Ex-CMPPy-GluA synthesized with PEG<sub>113</sub>-*b*-poly(GluA)<sub>10</sub>.

## 5. Chiral resolution by crystallization

### 5.1 Properties of chiral and racemic solids

One of the most fundamental and scientifically significant forms of chirality is the chirality of solids. The study of the crystallization of enantiomers and their packing arrangement in a crystal lattice led to many insights regarding their mutual interactions and elementary properties. Crystallization usually takes place in solutions. However crystals can be also obtained from molten or vapor phases. Crystals with a chiral property can be generally subdivided into two major groups. The first is crystals of non-chiral materials that adopt chiral crystal structures such as quartz and sodium chlorate. The second group consists of crystals of chiral molecules, i.e., enantiomers. Enantiomers can crystallize in three forms of racemic solids: conglomerates (racemic mixture), racemic compounds, and solid solutions called pseudo-racemate.<sup>118-119</sup> A racemic mixture is an equimolar physical mixture of the individual homochiral crystals of the two opposite enantiomers. A racemic compound consists of crystals in which the two enantiomeric molecules of opposite chirality are paired up in the unit cell of the crystal lattice. A pseudo racemate consists of the two enantiomeric molecules of opposite chirality arranged randomly in the same crystal lattice. The type of chiral solids found can be temperature dependent. A conglomerate can in principle be

mechanically separated. The most famous example is that of ammonium sodium tartrate. Enantiomerically-pure solids and racemic solids differ in their physical properties. These differences are not only important from a fundamental point of view, but also for the development of new approaches for the separation of enantiomers by crystallization.

Crystallization is one of the oldest methods known for the separation of enantiomers. Since the famous crystallization experiment of Pasteur, crystallization plays a central role in enantiomer resolution. Many strategies and models have been designed for the separation of enantiomers by crystallization. In general, the resolution of enantiomers by crystallization requires a racemate system that spontaneously resolves upon crystallization namely conglomerates. The classical method for the separation by crystallization incorporates diastereomeric transformation and chiral seeding. In the last two decades, there has been a new revival in the field of enantiomeric resolution by crystallization initiated by the pioneer research on racemic separation by 'tailored made additives'.

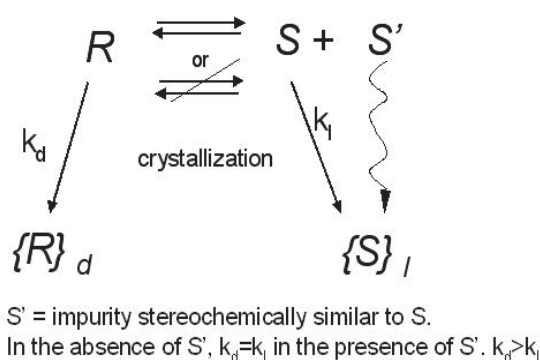
### **5.2 Chiral resolution by crystallization in the presence of soluble additives**

Additives are compounds that are structurally very similar to the crystallizing material; these molecules are referred to as guest molecules.<sup>120-121</sup> The additives are usually adsorbed onto specific crystal faces in a certain way that disrupts their continuous growth, and as a result these crystal faces grew in size. Morphological changes caused by additives that are similar in structure can be divided into two major cases, the inclusion of a 'disrupter' guest molecule or the inclusion of a 'blocker' guest molecule on the growing host crystal. In both cases the part of the guest molecules that structurally resemble the host molecules adsorbed on the crystal surfaces. A 'disrupter' molecule lacks certain functional groups that are essential for the growth of the crystal lattice, and thus creates vacancies in the crystal structure. A 'blocker' molecule is usually a functional group that is bulkier than the host molecules, thus causing steric interruption and preventing the continuous growth of specific crystal surfaces. In both cases the crystal growth is affected and the crystal habit is modified.<sup>121-122</sup> It should be mentioned here that the solvent molecules act similarly to additives to some extent, and therefore, crystallization in different solvents or solvent mixtures normally leads to the precipitation of crystals with different growth morphologies.<sup>123-130</sup> Polymers often serve as additives for controlled crystallization. The use of polymers as surfactants, morphology modifiers, or soft templates for crystallization is inspired exclusively by the biomineralization process. The attempt to mimic the biomineralization process led to the more extensive use of synthetic polymers, as well as biopolymers, in many crystallization processes for a wide range of applications. The control of crystallization by soluble polymeric additives relies mainly on a mutual recognition of the polymer functional groups and a certain location at the crystal surface similar to low molecular weight additives. This recognition determines the final structure of the crystals. The modification of crystal shape and morphology that occurs as a natural process is not a simple process, and apart from the interactions of the soluble polymers and the crystal surfaces there are other parameters that influence the final properties and structure of the crystals. Thus, synthetic polymers with known building blocks composition and a known behaviour in solvents can contribute to the study of such processes as a basic model.

### **5.3 Crystallization in the presence of synthetic polymers - resolution of enantiomers by 'tailor-made additives'**

The discussion of the chiral resolution of enantiomers by bio-inspired polymeric additives dates back to the pioneer research of Lahav and Leiserowitz who developed the resolution of

conglomerate crystals with 'tailor-made additives'.<sup>131-136</sup> They found that the incorporation of a small amount (up to a few percent) of enantiomerically-pure additives in the crystallization of chiral systems inhibits the crystallization of one enantiomer dramatically. In their study, Lahav and Leiserowitz encountered the interesting phenomenon of asymmetric induction on the crystallization of non-chiral photopolymerizable dienes into chiral crystals. The inducing agents were enantiomerically pure topochemical dimers, trimers, and oligomers of the same dienes. In all crystallization experiments the enantiomorphic crystal with an absolute configuration opposite to the enantiomorph of which the additive derived crystallized in excess. The authors established that the additives, which are stereochemically similar to the crystal from which they had been generated, adsorbed stereoselectively (in amounts of 1-2%) on the same enantiomorph, thereby inhibiting its growth. Consequently, the enantiomorph of opposite chirality precipitates in excess. It is known that the adsorption of small amounts of impurities on the surface of growing crystals may decrease their crystallization rates by several orders of magnitude. A natural extension of this hypothesis leads to the formulation of a general method for the resolution of enantiomers crystallizing in the form of racemic conglomerates. This process is illustrated in Scheme 2, where  $S'$  is an additive with stereochemistry similar enough to that of the unwanted enantiomer,  $S$ , to be adsorbed on its surface, but sufficiently different to disturb its further growth once adsorbed. For convenience, this process is named the 'rule of reversal'.



Scheme 2. General schema describes the rule of reversal<sup>132</sup>.

#### 5.4 Enantioselective crystallization by chiral soluble polymers

Zbaida and Lahav<sup>133,137-138</sup> continued their research on chiral resolution by crystallization with "tailor-made" additives and extended it from low molecular-weight additives to polymeric-resolving agents. Zbaida described the resolution by the crystallization of a series of racemic compounds such as glutamic acid, threonine, asparagine monohydrate, and histidine, using soluble chiral polymers. In a later publication, Zbaida demonstrated the use of soluble polymers based on R/L-lysine residues for the chiral separation of a lamellar twinning racemate conglomerate of valine and methionine.<sup>133</sup>

In a pioneer research, Mastai and Cölfen<sup>139</sup> presented chiral crystallization in the presence of DHBCs soluble chiral polymers in order to control chirality and to achieve racemate separation upon crystallization. In their study, a set of optically-active DHBCs were synthesized based on one polymer of PEG-*b*-PEI block (PEG poly(ethylene glycol),  $M_w = 5,000 \text{ g mol}^{-1}$ , PEI branched poly(ethyleneimine),  $M = 700 \text{ g mol}^{-1}$ ). In order to achieve chirality properties of the polymers as a natural occurrence, chiral molecules such as (S)-

ascorbate (vitamin C), (S)-proline, or (R)-gluconate were attached to the PEI amino branches. The different optically-active groups attached are summarized in Figure 5.1. Chiral moieties were attached to PEI throughout two main routes. In the case of amino-acid peptide-coupling methods and other chiral moieties, a nucleophilic attack by the PEI amine groups on the desired molecules was performed. The authors demonstrated the chiral crystallization for conglomerate systems of sodium ammonium tartrate ( $\text{NaNH}_4\text{T}$ ) and also for racemic systems of calcium tartrate tetrahydrate ( $\text{CaT}$ ). In the crystallization of  $\text{CaT}$ , it was found that appropriate chiral DHBCs can slow down the formation of the thermodynamically most stable racemic crystals as well as the formation of one of the pure enantiomeric crystals. Therefore, chiral separation by crystallization occurs even when racemic crystals are thermodynamically favored. However, chiral separation was only observed (though still with low chiral purity) at the early stages of the crystallization reaction. At longer crystallization times, the enantiomeric separation decreased again in favor of the racemate crystal formation. The presence of DHBCs can also modify the crystal morphology and create unusual morphologies of higher complexity, reflecting the polymer crystal interactions.

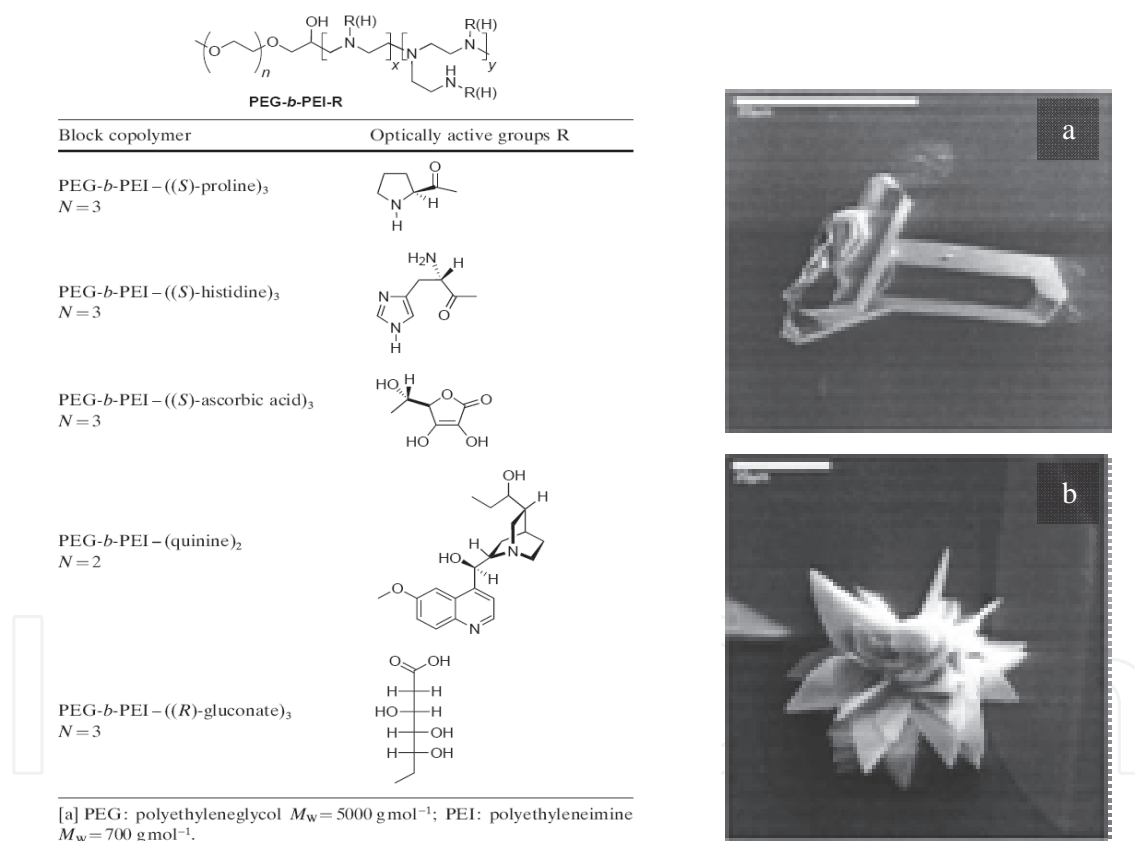


Fig. 5.1. Chiral copolymers used in the crystallization of racemic  $\text{CaT}$  and conglomerate  $\text{NaNH}_4\text{T}$ . ( $N$ =number of optically-active units). Crystal morphology of: a) conglomerate  $\text{NaNH}_4\text{T}$  formed in the presence of  $4 \text{ mg mL}^{-1}$  PEG-*b*-PEI  $\pm$  (S)-ascorbate; b) racemic  $\text{CaT}$  formed in the presence of  $15 \text{ mg mL}^{-1}$  PEG-*b*-PEI  $\pm$  (S)-proline (scale bar =  $5 \mu\text{m}$ ).<sup>139</sup>

Based on Mastai's findings, Menahem *et al.*<sup>140</sup> studied extensively the crystallization of racemic and conglomerate systems in the presence of soluble chiral polymers based on N-acrylic amino acid monomers. In Menahem's study, a series of poly-N-acrylic amino acid were synthesized by a method that involves the radical polymerization of N-acryl amino-

acid monomers leading to a polymeric acrylated amino-acid chain. These polymers are largely composed of amino acids, and hence they are highly water soluble. Menahem *et al* synthesized pairs of chiral poly-N-acryl amino acids based on D/L serine, D/L phenylalanine and D/L- Leucine amino acids [see Table 1].

Chiral Polymer D or L	<sup>1</sup> H NMR (ppm) (300 MHz)	Molecular Weights (g/mol)	Elemental Analysis (%)	[α] <sub>D</sub> <sup>24</sup>
Serine	(D <sub>2</sub> O): 1.59 (2H, broad), 2.35 (1H, broad), 3.8 (2H, broad), 4.3 (1H broad)	L-Ser = 27,600; D-Ser = 26,100	H: 6.549; C: 44.439; N: 7.615; O: 41.397	+16.2
Phenylalanine	(MeOD): 1.3 (2H, broad), 2.1 (1H, broad), 2.9 (2H, broad), 4.6 (1H, broad), 7.3 (5H, broad)	L-Phe = 280,700; D-Phe = 284,700	H: 6.769; C: 64.589; N: 5.727; O: 22.915	+24.1
Leucine	(DMSO): 0.87 (6H, broad), 1.53 (2H, broad), 3.6 (2H, broad), 4.2 (1H, broad)	L-Leu = 47,000; D-Leu = 47,300	H: 8.573; C: 58.024; N: 6.644; O: 26.759	+72.5

Table 1. Structure and Physical Data of Poly(N-acryl)amino acids<sup>140</sup>

These polymers were tested as promoters of enantioselective crystallization in the chiral crystallization of conglomerate and racemates. In a typical crystallization experiment, a minimal amount of chiral soluble polymers (1-5mg/mL) was added to the crystallizing system. To perform chiral crystallization, racemates and conglomerate crystal systems of amino acids were chosen. D, L-asparagine hydrate, D, L-threonine, and D, L-methionine served as model systems for conglomerate crystallization. DL-histidine and D, L-glutamic acid served as a characteristic case of racemic compound crystallization. The results of the variety of crystallization experiments performed are summarized in Table 2. In different crystallization experiments of D, L-histidine and D, L-glutamic acid in the presence of each of the chiral polymers (1-5mg/mL) no influence on the chirality was observed, meaning that the enantiomeric excess (ee) measured during and at the last stages of crystallization was zero. Although no chiral selective induction was observed for the racemates crystallization, the chiral polymers interacting with the racemic solid phase result in an evident change in crystal morphology (Figure 5.2).

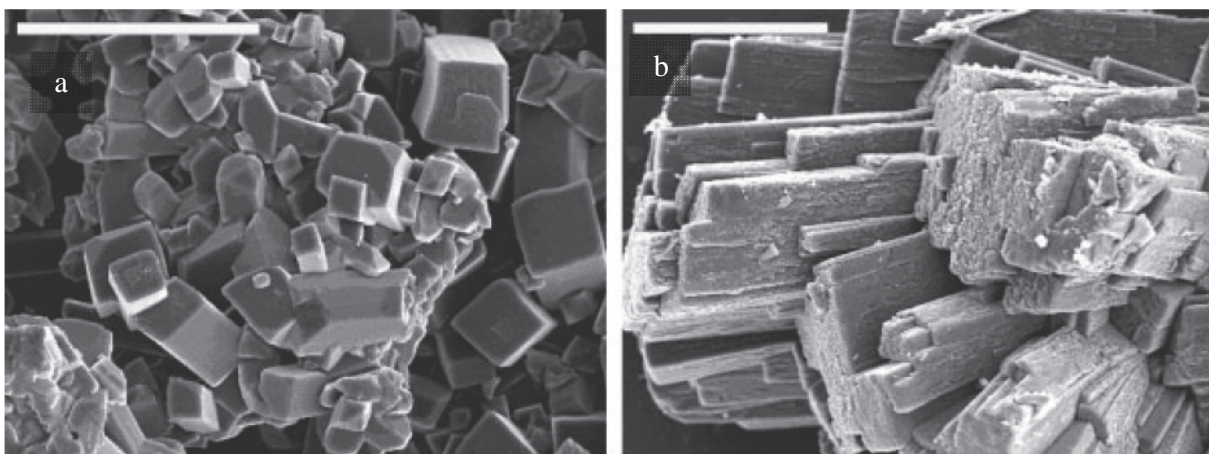


Fig. 5.2. SEM images of DL-histidine crystals: (a) Crystal morphology of D, L-histidine crystallized from pure water (no chiral polymer additives) and (b) Crystal morphology of D, L-histidine formed in the presence of 1 mg/mL of poly-L-phenylalanine (scale bar = 100 μm).<sup>140</sup>



The crystallization experiments for conglomerate systems in the presence of soluble chiral polymers led the authors to two major conclusions. Primarily, these chiral polymers were found to be excellent resolving agents for kinetic resolution by crystallization for conglomerate systems. For example, analyses of the results of the crystallization of D, L-threonine in the presence of 1 mg/mL of poly-L-leucine verify high chiral discrimination at the early crystallization stages; an e.e. of about 85% was recorded within the first stage of crystallization [Table 3]. Second, a certain chiral polymer will not routinely resolve structurally-related compounds and correspond to the rule of reversal, meaning that the rule of reversal does not necessarily hold true.<sup>141</sup> For example, resolution experiments of D, L-methionine with poly-D-serine and with poly-L-serine show an e.e of about 30% of the same isomer, namely, the L crystal form of D, L-methionine. Similar results are also observed for the crystallization of D, L-threonine with chiral polymers of D- and L-phenylalanine [Table 2].

Conglomerate	Polymer (1 mg/mL)	Crystal Yield <sup>a</sup> (%)	e.e <sup>b</sup> (%)
Threonine	No polymer	Under all conductions	1.25 D
Threonine	L-Leucine	6.7	84.3 D
Threonine	L-Leucine	26.7	46.6 D
Threonine	L-Leucine	46.7	18.2 D
Threonine	L-Leucine	68.1	2.59 D
Threonine	L-Phenylalanine	21.6	15.3 L
Threonine	D-Phenylalanine	28.6	11.3 L
Asparagine hydrate	L-Leucine	13.6	87.2 D
Asparagine hydrate	L-Leucine	33.7	41.5 D
Asparagine hydrate	L-Leucine	71.5	3.7 D
Asparagine hydrate	D-Leucine	18.2	52.5 L
Methionine	D-Serine	20.3	29.7 L
Methionine	L-Serine	23.8	23.4 L

Table 2. Typical Resolution Experiments at Room Temperature of Conglomerate Crystals with Chiral Polymers.

New insights on the relation between the absolute configuration of the soluble copolymer and the crystallization of chiral amino acids were recently published by Menahem *et al.*<sup>142</sup> In this report the authors draw a correlation between chiral polymer structures, particularly  $\alpha$ -helical and random coil conformations, and their efficiency as chiral resolving agents in chiral crystallization processes. For that reason, DHBCs based on polyethylene oxide (PEO) with chiral glutamic acid blocks (PEG<sub>113</sub>-*b*-(+)-(S)-Glu<sub>20</sub>) and its correspond (PEG<sub>113</sub>-*b*-(-)-(R)-Glu<sub>20</sub>) were synthesized. The chiral block copolymers were synthesized via the ring opening polymerization of a (+)-(S)-glutamic acid N-carboxyanhydride monomer with PEO (Mw=5,000 gr/mol) as a macroinitiator. Their secondary structure at different pHs was determined by Circular Dichroism (CD) measurements [Figure 5.3]. Synthetic peptides can adopt three main types of secondary structures,  $\alpha$ -helix,  $\beta$ -sheet, and random coil. The absolute secondary structure depends on the polymer's solute conditions, mainly pH or temperature. At low acidic pH, the amino acid block shows a CD spectrum typical for an  $\alpha$ -helix. At basic pH, the secondary structure of the peptide block was a random coil (100%)

and at pH values between natural pH, mixtures of both structures were found with increasing contents of  $\alpha$ -helix with decreasing pH.

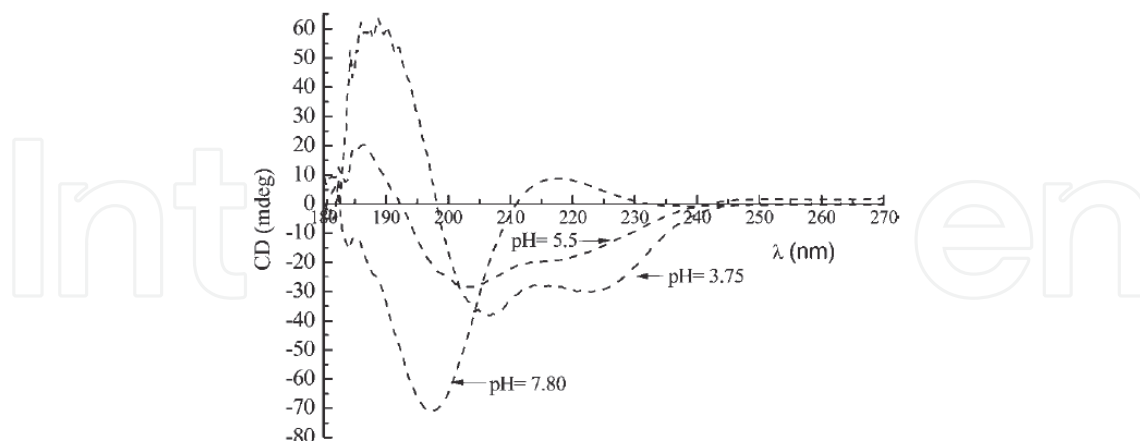


Fig. 5.3. Circular dichroism measurements of PEO<sub>113</sub>-*b*-(+)-(S)-Glu<sub>20</sub> at different pH at 25°C.

All crystallization experiments were conducted from supersaturated solutions of threonine. The crystallization experiments of threonine were performed at two pH values: pH 3.75 and pH 7.80. The chiral polymers in the different conformers participated in the chiral crystallization of D, L threonine [summarized in Table 1]. The DHBCs polymers were used as soluble additives in the crystallization reaction. In their report, the authors stated that PEO<sub>113</sub>-*b*-(+)-(S)-Glu<sub>20</sub> and PEO<sub>113</sub>-*b*-(-)-(R)-Glu<sub>20</sub> showed the identical behaviour and influence on the crystallization experiment. The polymers influenced the crystallization kinetics, crystal morphology, and chiral resolution of D, L threonine. Regarding chiral resolution, the conclusion arising from this report is that the chiral resolution of D, L-threonine take place only in the presence of PEO<sub>113</sub>-*b*-(+)-(S)-Glu<sub>20</sub> in its  $\alpha$ -helix form, i.e., in acidic conditions [Table 3]. For example, the presence of 1 mg/mL of PEG<sub>113</sub>-*b*-(+)-(S)-Glu<sub>20</sub> at pH 3.75 verifies the chiral discrimination of about 19.1% e.e (of the R enantiomers) at the early crystallization stages. The results of chiral resolution by crystallization show a linear correlation between the polymer concentrations in solution and the resolving power of the polymer. At higher polymer concentrations, a high e.e. was achieved. For instance, at a polymer concentration of 0.1 mg/mL, a maximum e.e. of 20.9% is achieved, whereas at similar conditions with a polymer concentration of 1 mg/mL, an e.e. of 35.8% was achieved.

pH	Chiral polymers (mg/ml)	Crystal yield (%)	e.e.
Exp. conditions			
3.75	1 mg/ml PEO <sub>113</sub> - <i>b</i> -(+)-(S)-Glu <sub>20</sub>	6.8	19.1 (R)
3.75	1 mg/ml PEO <sub>113</sub> - <i>b</i> -(+)-(S)-Glu <sub>20</sub>	10.2	35.8 (R)
3.75	0.5 mg/ml PEO <sub>113</sub> - <i>b</i> -(+)-(S)-Glu <sub>20</sub>	10.2	29.2 (R)
3.75	0.1 mg/ml PEO <sub>113</sub> - <i>b</i> -(+)-(S)-Glu <sub>20</sub>	5.4	11.9 (R)
3.75	0.1 mg/ml PEO <sub>113</sub> - <i>b</i> -(+)-(S)-Glu <sub>20</sub>	9.1	20.9 (R)
7.80	1 mg/ml PEO <sub>113</sub> - <i>b</i> -(+)-(S)-Glu <sub>20</sub>	7.7	4.4 (R)
7.80	1 mg/ml PEO <sub>113</sub> - <i>b</i> -(+)-(S)-Glu <sub>20</sub>	10.9	3.1 (R)
7.80	0.5 mg/ml PEO <sub>113</sub> - <i>b</i> -(+)-(S)-Glu <sub>20</sub>	3.5	2.8 (R)
7.80	0.5 mg/ml PEO <sub>113</sub> - <i>b</i> -(+)-(S)-Glu <sub>20</sub>	9	3.7 (R)
7.80	0.1 mg/ml PEO <sub>113</sub> - <i>b</i> -(+)-(S)-Glu <sub>20</sub>	5.3	3.1 (R)
7.80	0.1 mg/ml PEO <sub>113</sub> - <i>b</i> -(+)-(S)-Glu <sub>20</sub>	8.7	1.1 (R)
Control exp.			
3.75	1 mg/ml PEO <sub>113</sub>	8.1	2.2 (S)
7.80	1 mg/ml PEO <sub>113</sub>	6.1	3.1 (R)

Table 3. Resolution experiments at room temperature of D, L-threonine with chiral polymers.

### 5.5 Crystallization in the presence of insoluble chiral microspheres

In nature not only soluble natural polymers are important for shaping crystal properties, and an insoluble matrix can also dramatically influence the growth of a biomaterials.<sup>25</sup> Therefore, chiral-insoluble supports have attracted a lot of attention recently for their possible use as a chiral auxiliary for chiral resolution by crystallization. Using insoluble particles with a chiral character as additives in the crystallization process provides chiral surfaces on which crystallization is favoured.

As mentioned before, chiral particles were synthesized in many versions and techniques for a variety of applications, but mainly as a solid support for chiral separation by chromatographic methods. Two of the most prominent methods for the preparation of chiral particles are molecular chiral imprinting and chiral coating of silica or polymeric particles with chiral species. Although both methods suffer from many drawbacks, they are still extensively used. Margel and Melamed have suggested a new approach for the synthesis of polymeric spheres made of *N*-vinyl *α*-phenylalanine monomer polymerized into non-crosslinked and crosslinked poly(*N*-vinyl *α*-phenylalanine) microspheres.<sup>143-144</sup> The non crosslinked particles were formed by the dispersion homopolymerization of the vinyl monomers in different mixtures of water and 2 propanol. Under the polymerization conditions equilibrium exists between insoluble polymer composed of poly (*N*-vinyl-*α*-phenylalanine) particles and its corresponding soluble polymer. In order to avoid the formation of soluble polymeric chains, the authors illustrated the synthesis of crosslinked spheres using dispersion polymerization. Spherical particles prepared by the latter method were used to bind biological impotent macromolecules such as trypsin peptide. Through this method, Menahem *et al.*<sup>140</sup> polymerized chiral amino acid L/D phenylalanine acryl monomers in a dispersion polymerization procedure. As presented by Margel *et al.* the formed particles were used as insoluble additives in chiral crystallization experiments of racemates, D, L-histidine and D, L-glutamic acid, as well as in the crystallization of conglomerate systems of D, L-asparagine hydrate, D, L-threonine, and D, L-methionine. In a typical experiment, insoluble particles (0.1-5 mg/mL) were added to a supersaturated solution of each amino acid. The crystallization proceeds in the presence of the chiral particles and the crystallization process ended with the precipitation of the crystals. Optical measurements were taken during all the crystallization processes to track the enantiomeric excess. In the crystallization experiments with racemates, the chiral polymers didn't show any chiral resolution ability. However, the crystallization of the conglomerate system DL-threonine, in the presence of the chiral poly L-phenylalanine microspheres showed significant chiral recognition during the crystallization process. Time resolved polarimetry revealed that the maximum chiral recognition occurs after 22 hours of crystallization. At the end of the crystallization process, a decrease in the resolution ability was recorded. Scanning Electron Microscopy ((SEM) analysis showed that crystals were grown on the surface of the chiral particles with significant changes in morphology [Figure 5.4].

We recently introduced the next generation of chiral polymeric microspheres, and presented a new approach to the synthesis.<sup>145-146</sup> This new path resulted in well-defined morphology, porosity, roughness, and microparticle size that is wholly chiral. In this new development, chiral *N*-acryl- L-phenylalanine monomers were polymerized via a one-step swelling dispersion polymerization process.<sup>147-149</sup> The one-step swelling process was developed by Margel *et al.*<sup>150-152</sup> In this method swelled polystyrene particles served as the reaction vessel, and therefore the first step is the polystyrene swelling process. Non crosslinked polystyrene particles were added to a microemulsion of organic solvent (chlorobromobenzene or

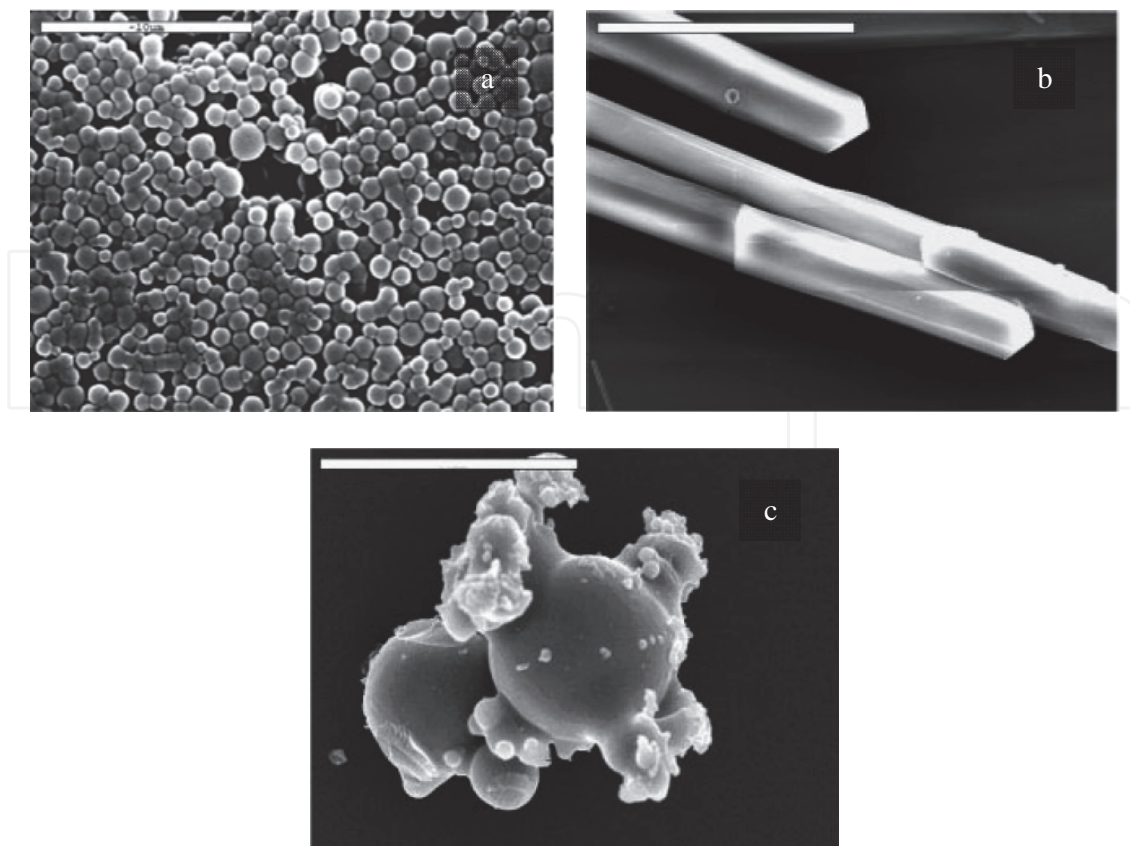


Fig. 5.4. SEM images of (a) poly-D-phenylalanine microspheres formed by precipitating in water/2-propanol ratio of 0.1.(b) Crystal morphology of D, L-threonine crystallized from pure water (no chiral polymer additives), (c) chiral microsphere of poly-L-phenylalanine collected after crystallization of DL-threonine (scale bar = 10  $\mu\text{m}$ )<sup>140</sup>.

chlorobenzene) droplets in water. water. The polystyrene particles merged with the organic droplets, which led to their swelling. In a sequential step chiral monomers, initiator and a crosslinker (divinyl benzene) were dissolved in the swollen polystyrene particle template. Polymerization initiated with the rise in temperature. After the polymerization, the polystyrene particles were dissolved by dry acetone. The outcome of this process was remarkable; mono-disperse microspheres of  $5.4 \mu\text{m} \pm 2\%$ , i.e., whole chiral, were obtained with a perfectly defined structure. The partial morphology was controlled by varying the polymerization conditions such as swollen solvents and monomer/crosslinker ratio. For example, the use of chlorobromobenzene as swelling solvent resulted in hollow particles while the use of chlorobenzene resulted in porous polymeric material [Figure 5.5].

Poly vinyl L-phenylalanine particles with a porous structure were used to test the chiral selectivity by enantiomeric adsorption from racemic and enantomerical pure D- and L-valine solutions. The polymeric particles showed chiral selective adsorption toward the L-valine enantiomer in time-resolved polarimetry experiments. Crystallization experiments of racemic D, L-valine in the presence of the chiral particles (10 mg/mL) resulted in enantioselective crystallization on the chiral hollow particles [Figure 5.6]. The crystallization experiment was quenched when a solid phase appeared on the particles surfaces that could be observed by a light microscope. In order to analyze enantioselective crystallization on the chiral hollow microspheres powder X-ray diffraction (XRD) and differential scanning calorimetry (DSC) were used. The X-ray diffraction pattern of pure valine enantiomers is

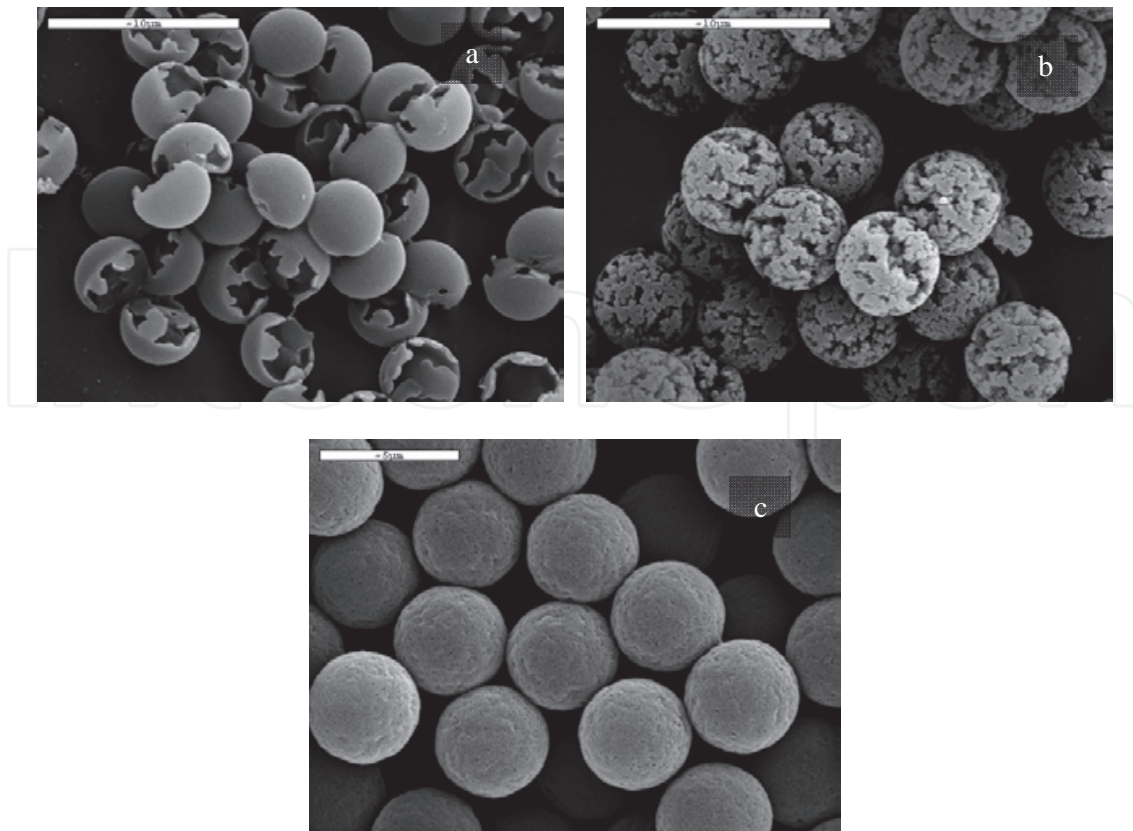


Fig. 5.5. SEM images of Poly L-phenylalanine microspheres of different polymerization conditions. (a) Hollow structure (b) Porous particles (c) Rough surface particles.<sup>145</sup>

different from that of the corresponding racemic compound owing to their different crystalline structures. The XRD pattern of valine crystals crystallized on chiral microspheres shows a typical set of diffraction peaks corresponding to D, L-valine, as well as two additional diffractions that match the diffraction from the (0,0,3) and (1,1,-3) crystal planes of the pure enantiomer of valine. The observation that the XRD patterns of D, L-valine crystals crystallized on the chiral microspheres display diffraction peaks corresponding to the pure enantiomer of valine clearly demonstrates that enantioselective crystallization has been achieved on the chiral microspheres. In addition, the thermal behaviour of the racemates and pure enantiomers is different and, accordingly, traces of enantiomers can be examined by DSC. Further evidence for the generation of pure enantiomeric crystals on the chiral microspheres is obtained from DSC measurements showing a shift of the melting point to lower temperatures ( $T=244^{\circ}\text{C}$ ), indicative of the presence of pure enantiomeric valine crystals. Although XRD and DSC clearly reveal enantioselective crystallization onto the chiral microspheres, it is difficult to obtain quantitative information about chiral separation from these techniques. However, optical rotation measurements during crystallization show an enantiomeric excess of ca. 25% for crystallization onto chiral microspheres.

Hybrid organic inorganic nanoparticles have been reported recently.<sup>153</sup> Chen *et al.* have reported on a novel approach for the synthesis of hybrid particles based on a chiral polymeric core of poly polyacetylene with a silica shell. The particles were prepared in a one-pot, two-step polymerization reaction. In the first step, acetylene monomers, N-propargyl-(1*S*)-camphor-10-sulfamide [(*S*)-SA], and N propargyl (1*R*)-camphor-10-

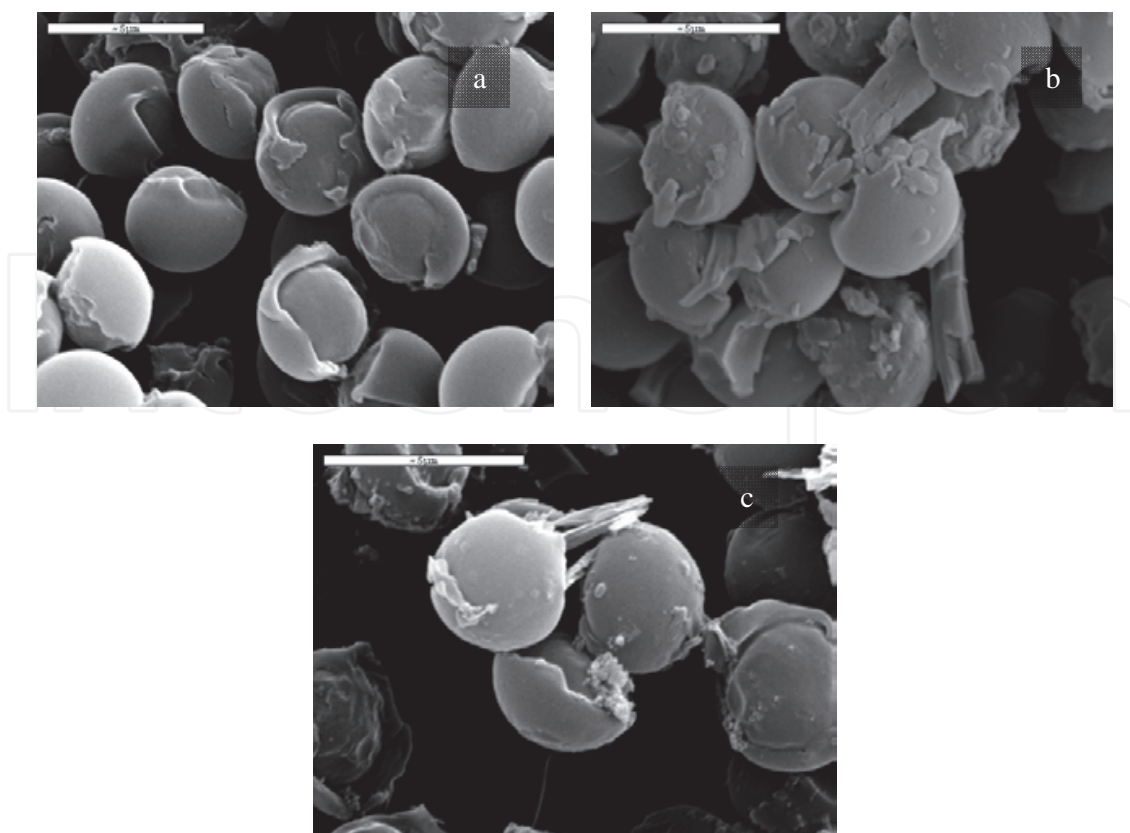


Fig. 5.6. SEM images of chiral PV-L-Phe microspheres after crystallization of D; L-valine (scale bars = 5  $\mu\text{m}$ ).<sup>145</sup>

sulfamide [(*R*)-SA], were polymerized in catalytic microemulsion polymerization to give the polymeric (*S*) PSA and (*R*) PSA, respectively, resulting in helical substituted polyacetylene. This stage provides the authors with the particle's core. In the second step, the shells were formed via a sol-gel approach of TEOS (tetraethyl orthosilicate) in the same aqueous system. The particles were shown to be optical active and the exhibited good thermal stability with regards to the same core particles with a polymeric shell instead of a silica core. These particles were used as chiral selectors for the chiral crystallization of the racemate system of DL-alanine. The obtained particle (*S*)-PSA and (*R*)-PSA were reported as chiral selectors toward D and L alanine respectively during chiral crystallization.

## 6. Antifreeze proteins

Freezing is almost always lethal to cellular organisms as it deprives biological processes of the aqueous medium they require, causes a concentration of ions and other solutes in the plasma, induces denaturing of biomolecules, and ruptures cell membranes. Despite this, polar and near-polar fish typically survive in seas where the temperature is subzero, frequently as low as  $-1.9\text{ }^{\circ}\text{C}$ . In a series of articles from 1953 through 1972, Scholander's group<sup>154-156</sup> observed abnormally low freezing temperature of blood serum from Arctic fish, and reported that this was not due to the presence of additional salts or other colligatively-acting substances. Publications, first by DeVries *et al.*<sup>157-159</sup> and later by others, described the existence of a glycoprotein in the sera of Antarctic fish that lowered the freezing temperature without increasing the osmotic pressure, which otherwise would have been

lethal to the fish. Since these early studies, there have been many reports on these and similarly acting proteins, some of which are not glycoproteins. Many reviews have appeared, some on the overall characteristics of these substances, others devoted to the mechanisms of functioning.<sup>160-165</sup>

Overall there are two main families of proteins that possess the capability of depressing the freezing temperature of water. They are the *antifreeze glycoproteins* (AFGPs) and the *antifreeze proteins* (AFPs), which show similar properties. The structures of both AFP and AFP have been extensively studied for several decades. Antifreeze glycoproteins and antifreeze proteins comprise several structurally-diverse classes of molecules that have in common the ability to inhibit the growth of ice. Antifreeze glycoproteins are carbohydrate rich (ca.  $2.6 \pm 34$  kDa) proteins containing an  $(\text{Ala-Ala-Thr})_n$  repeat unit with a disaccharide attached to threonine unit. At least four classes of structurally-independent antifreeze proteins have been identified: type I, alanine-rich,  $\alpha$ -helical 3.3 to 4.5-kDa proteins; type II, cysteine rich globular proteins that contain five disulfide bonds; type III, approximately 6 kDa globular proteins; and very recently, type IV, glutamate- and glutamine-rich proteins that contain  $\alpha$ -helices but appear to be unrelated to other proteins. In Table 4 the different types of AFPs and AFGPs: are shown.

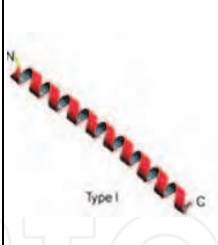

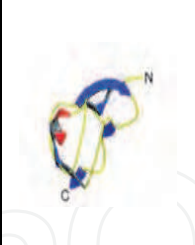

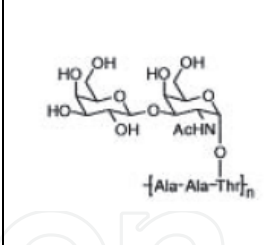
Properties	AFP - I	AFP - II	AFP - III	AFP - IV	AFGP
Molar Mass (Da)	4,500-3,300	24,000-1,100	6,500	12,000	33,000-2,600
Primary structure	Enriched with Ala	Enriched With Cys disulfide bonds.	Does not exhibit a characteristic structure	Enriched with Glu and Gln	$(\text{Ala-Ala-Thr})_n$ the hydroxyl side group
Secondary structure	$\alpha$ helix	$\beta$ sheet	$\beta$ sheet	Amphiphilic $\alpha$ -helix	Random
Representative structure					

Table 4. Structural characteristics of the antifreeze proteins and glycoproteins.

AFPs and AFGPs appear to have the capability of depressing the freezing point of water in some cases by several degrees at low concentration ( $10^{-3}$  M or lower). Therefore, these proteins act as non colligative antifreeze agents. In addition, AFPs and AFGPs show "thermal hysteresis" behaviour. In other words, they lower the nonequilibrium freezing point of water (in the presence of ice) below the melting point by a non-colligative mechanism, thereby producing a difference between the freezing and the melting points. This behavior reveals that the ice crystal growth mechanism is strongly connected to a kinetic effect on the ice/water surface being modified by adsorbing these proteins. It is widely accepted that molecules of AFPs are adsorbed onto an ice surface, and that the crystal growth of ice between the adsorbed molecules produces a microscopic curved surface. Since the energetic cost of adding a water molecule to this convex surface is high, a

non-equilibrium freezing point depression is observed, while the melting point remains constant. This is known as the Kelvin effect, and the difference between melting and freezing points is defined as thermal hysteresis. AFPs and AFGPs also inhibit the growth of ice crystals, and strongly retard re-crystallization due to their interactions with the ice crystal lattice.

The adsorption mechanism of AFPs to the ice crystal surface at the molecular level is still not clearly understood. Knight *et al.*<sup>165</sup> hypothesized that this adsorption is caused by hydrogen bonding between repeated hydrophilic amino acids and ice crystal lattices. However, Chao *et al.*<sup>166</sup>, Haymet *et al.*<sup>167</sup>, and Zhang *et al.*<sup>168</sup> replaced hydrophilic amino acids with other amino acids in type I AFP and showed that hydrogen bonding is not necessary for antifreeze effects. These results suggest that van der Waals and hydrophobic interactions are the main cause for the adsorption, and that the complementary fit between the ice binding surface of AFP molecules and ice surfaces is required for adsorption.

In addition, the adsorption mechanism of AFGPs to the ice crystal surface at the molecular level is still in dispute. Researchers suggested that the binding of AFGPs to the ice surface likely involves hydrogen bonding between the polar groups of the saccharide residue (the hydroxy groups) and the ice surface. However, other studies have demonstrated that the number of potential hydrogen bonds between the antifreeze molecule and the ice surface appears to be insufficient to explain the observed tight binding of AFGPs to ice. Modeling studies have looked at all possible binding configurations, and in the best case only two hydroxy groups per disaccharide are in a position to form hydrogen bonds with the ice surface. Each hydroxy group forms only one hydrogen bond with the ice surface. In AFGP 8 (with four glycosylated tri peptide units), this would allow only eight hydrogen bonds with the ice surface. Consequently, it is difficult to explain how the adsorption of AFGP 8 onto the ice surface is irreversible. In an attempt to rationalize this irreversible binding of AFGP 8, Knight *et al.*<sup>169</sup> proposed an alternate model. In their model, the hydroxy groups of the disaccharide are actually incorporated into the ice lattice. In this fashion, each hydroxy group is able to form three hydrogen bonds within the ice lattice. Assuming that in each disaccharide only two hydroxy groups are able to interact with the ice surface, this allows AFGP 8 a total of twenty-four hydrogen bonds to the ice surface instead of eight, and may explain why adsorption is irreversible. Similar to the AFPs, AFGP researchers have been divided over the importance of hydrogen bonding and its role in the mechanism of action. While it has been proposed that the hydrophilic interactions between polar hydroxy groups and the water molecules on the ice surface are extremely important,<sup>170</sup> others have invoked the idea that entropic and enthalpic contributions from hydrophobic residues are crucial in the binding of an AFGP to an ice surface. Despite the fact that significant entropic contributions are likely to be gained upon the exclusion of water from the protein and ice surfaces, a definitive mechanism invoking hydrophobic and/or hydrophilic interactions with emphasis on the role they play in adsorption of the antifreeze to the ice surface has failed to emerge.

Although AFPs and AFGPs hold great promise for various biotechnology applications, significant problems have prevented them from being developed commercially. For instance, they are relatively expensive, easily degraded by bacteria, difficult to purify and synthesize, and chemically unstable in solutions. Therefore, the development of cheap and stable substitutes for AFPs and AFGPs is necessary.



### 6.1 Mimicking AFP and AFGP behavior for antifreeze applications

The general concept in biomimetic polymers for antifreeze application is to design polymers that can mimic the structures and functionality of antifreeze proteins. In light of this, a few studies sought to mimic the structure and function of AFPs and AFGPs for antifreeze applications. Inada *et al.*<sup>171</sup> was the first to investigate the use of silane coupling agents (SCAs) as substitutes for AFPs. In their study, they observed the free growth of ice crystals in SCA solutions and found that SCAs that form long-chain molecules in water are effective for ice crystallization control. They then analyzed ice crystal surfaces containing SCAs using scanning tunneling microscopy (STM) to investigate the mechanism of crystallization control with these additives. STM observations showed the existence of grooves on the surface of ice crystals produced by the SCAs at intervals of several hundred nanometers. These results suggest that the molecules of these additives can be adsorbed on ice surfaces as well as AFPs, thus inhibiting further crystal growth between the adsorption sites by the Kelvin effect.

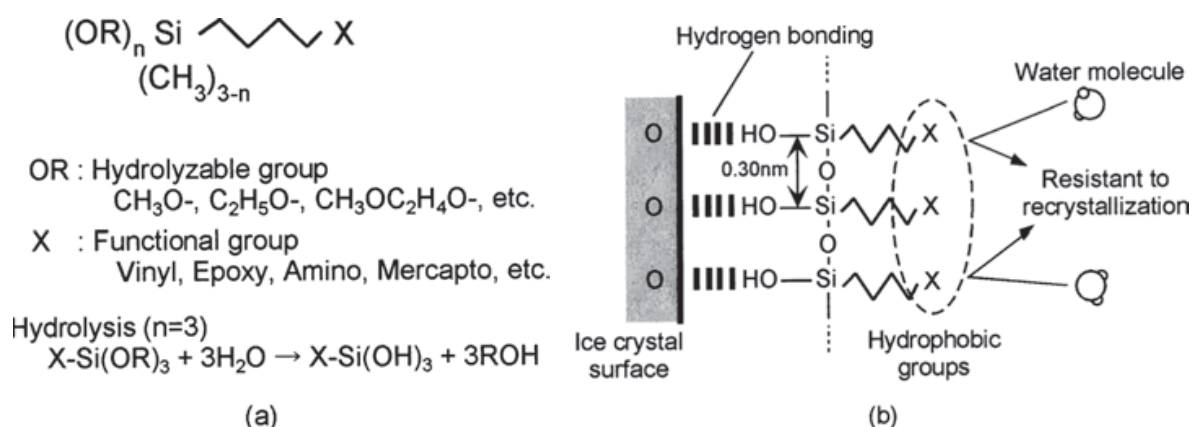


Fig. 6.1. Properties of SCA: (a) Structural formula of SCA and its hydrolysis process; (b) Model of long-chain SCA molecules adsorbed on an ice crystal surface.

Polyvinyl alcohol and related compounds are shown to inhibit the freezing of water and water solutions. These synthetic compounds preferentially bind and inhibit ice-nucleating surfaces in a manner similar to natural antifreeze proteins. Inada *et al.* and Lu *et al.*<sup>172</sup> investigated the surface morphology of ice crystals containing adsorbed PVA molecules at  $-7.0$  °C by STM. PVA was used as a substitute for type I AFP, which is an effective additive for making ice slurries resistant to recrystallization. The STM images revealed microscale grooves on ice crystals made from PVA solutions, indicating that PVA molecules significantly influence the surface structure of the ice crystal. The length of each groove was similar to that of a PVA molecule, indicating that these molecules were adsorbed on the ice crystal surface. The interaction force between PVA molecules and the ice surface was discussed by assuming a molecular structure of PVA on the ice crystal surface, as shown in Figure 6.2. The depression of the local freezing point was analyzed based on the surface curvature of ice revealed by STM.

In addition, these researchers reported<sup>173</sup> evidence of the thermal hysteresis caused by PVA. Thermal hysteresis is often taken as the primary manifestation of the antifreeze activity of biological non-equilibrium antifreezes, such as AFPs and AFGPs. Similar to these molecules, PVA molecules stopped the growth of ice in the melt even at temperatures below the melting temperature of ice, although they exhibited very slight thermal hysteresis compared

with most known biological antifreezes. The crystal habit of ice in the melt in the presence of PVA indicated that PVA molecules affected specific planes of the ice crystal.

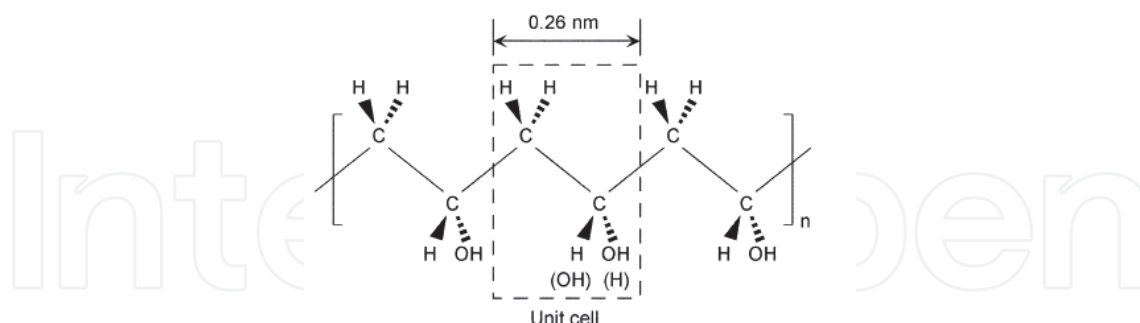


Fig. 6.2. Molecular structure of PVV showing a unit cell enclosed by the broken line contains two hydroxyl sites, each with 50% occupancy.

Mastai *et al.*<sup>174</sup> found that block copolymers based on a poly(ethylene oxide) block and a poly[2-(2-hydroxyethyl)ethylene] block with functional groups analogous to AFPs can influence the bulk structure of liquid water. The chemical structures of those block copolymers are shown in Figure 6.3.

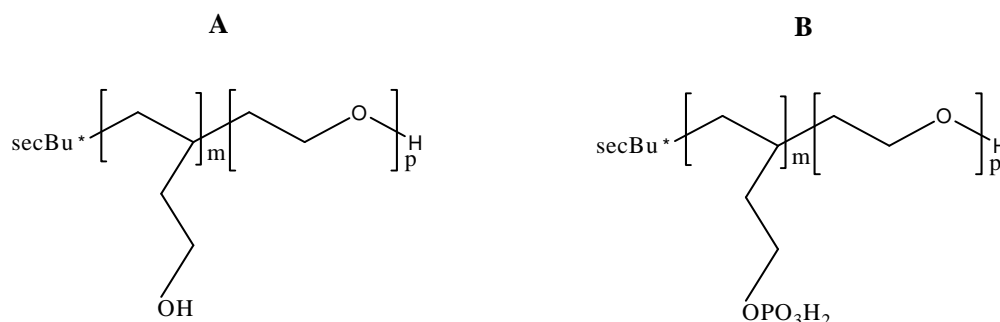


Fig. 6.3. Structures of the "antifreezes" block copolymers (A) poly(ethyleneoxide)-block-poly[2-(2-hydroxyethyl)ethylene] (PEO-b-PHEE) and (B) a partially (30%) phosphorylated poly(ethyleneoxide)-block-poly[2-(2-hydroxyethyl)ethylene] (PEO-b-PHEE-OPO<sub>3</sub>H<sub>2</sub>).

Those block copolymers shown to be effective in inhibiting the recrystallization of ice. Evidence for their antifreezes activities is given by various experimental methods for example: an excess increase of the bulk water density, strong water viscosity changes, additional exothermic enthalpic transitions close to the freezing point, as well as changes in the ice unit cell. The strong effect of the block copolymers on the morphology of ice crystals and inhibition of ice crystallization is nicely demonstrated in recrystallization experiments. In recrystallization experiments a drop of pure water or block copolymer solutions is placed onto a cooled microscopy glass plate (190 K), and an amorphous ice film formed instantly. The ice film is then warmed to 265 K and allowed to recrystallize for 2 hours. Light microscopy with a temperature controlled cold stage is used to investigate the crystal morphology and size and the results of such experiments are shown in Figure 4. For pure water crystals grow to larger units (Figure 6.4 a), while in the presence of the block copolymers smaller ice crystals are found, which keep their original size and morphology even after longer growth times (Figure 6.4 b). This inhibition of re-crystallization is indicative of a strong interaction of the block copolymer with the ice surfaces and a lowering

of the surface tension of the exposed planes. Therefore, the block copolymer effectively suppresses Ostwald ripening and stabilizes small ice microcrystals.

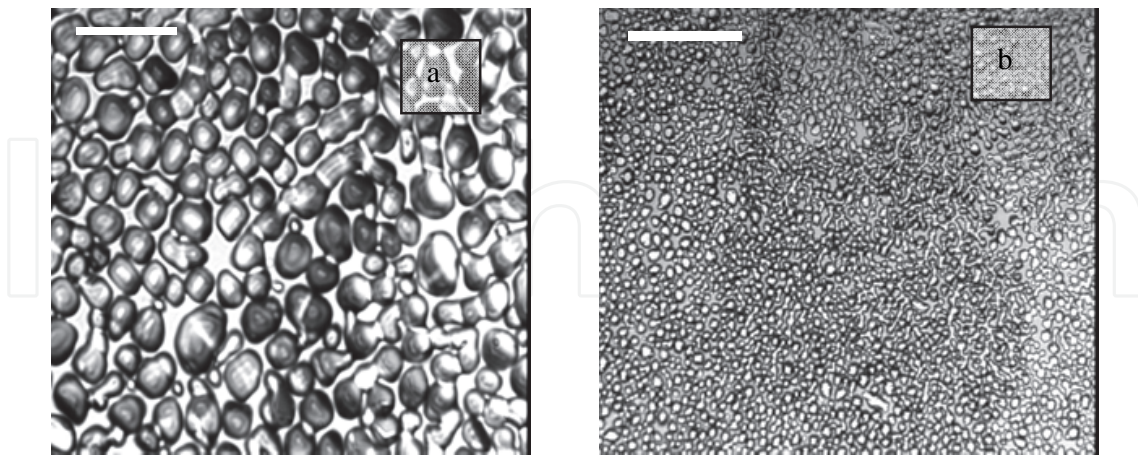


Fig. 6.4. Ice recrystallization experiments. (a) Pure water, (b) water with 25 mg/mL polymer of PEO-*b*-PHEE Scale bars 3  $\mu\text{m}$ .

Other classes of polymers that have been tested for antifreeze application are based on linear polyglycerol. Funakoshi *et al.*<sup>175</sup> employed linear polymers of glycerol in combination with other ice control agents, such as PVA, polyvinyl acetate copolymers and AFPs to provide anti-nucleation effects that were superior to those of either polyglycerol or the co-anti-nucleator alone. Polyglycerol has a number of advantageous physical and toxicological properties, such as extreme water solubility, non-toxicity to humans, non-toxicity to animal tissues *in vitro* even at extreme concentrations, minimal foaming tendency, minimal retention on hydrophobic surfaces, and stability in solution without the need for periodic heating to reactivate its anti-nucleation properties.

Further study by Baruch *et al.*<sup>176</sup> demonstrated that hyper branched copolymers containing poly(ethylene oxide)-polyethyleneimine blocks and polyglycidol side chains exhibit antifreeze properties. A modular set of block copolymers with hydroxyl groups as functional groups were synthesized. The polymer synthesis was restricted to double block copolymers with relatively small block lengths; for the poly(ethylene oxide) (PEG) block polymer with average Mw of 5,000 gr/mol were used and for the polyethyleneimine (PEI) polymer block with average length of Mw 2,000 gr/mol were used. The attachment of glycidol side chains to the PEI block is performed by reaction with diglyme. The antifreeze properties of hyperbranched polyglycidol polymers were investigated by several techniques such as DSC, nanoliter osmometry, XRD, and optical microscopy. It has been demonstrated that the hyper-branched copolymers of polyglycidol can lead to a strong freezing point depression of water to 0.8 °C at a relatively low concentration (1 mg/mL). It is also shown that hyper-branched polyglycidol influences of the crystallization kinetics of ice (slowing down) and leads to changes in the ice crystal morphology.

Recently Yagci *et al.*<sup>177</sup> showed that multifunctional poly (tartar amides) polymers can strongly interfere with the crystallization process of water in comparison with commercially available commodity polymers. While the addition of the poly(tartar amides) results in a minor freezing point depression, as is shown by differential scanning calorimetry, a strong change in the ice crystal morphology is evident. Wide-angle X-ray scattering and optical microscopy indicated (Figure 6.5) that the hexagonal structure of undisturbed ice-crystals was oriented and partly deformed.

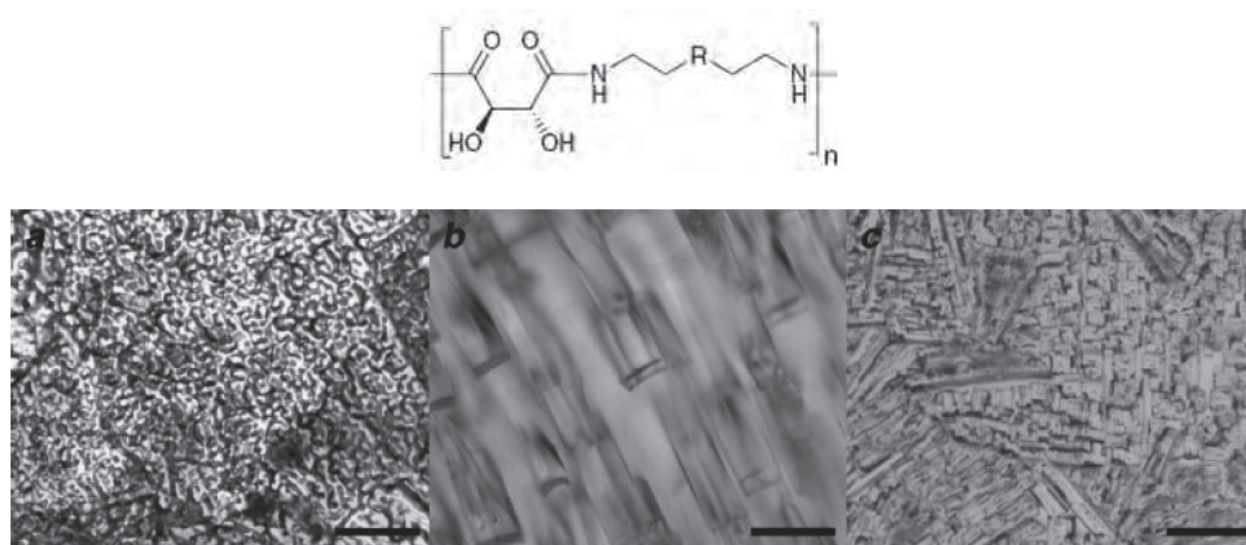


Fig. 6.5. (I) Chemical structure of the poly (tartar amides) polymers. Light microscopy images of ice crystals at 223 K after freezing assay, (a) pure water, (b+c) polymer solutions of poly(tartar amides) at concentration of 2 wt.-%, scale bar equals 100  $\mu$ m).

Gibson *et al.*<sup>178</sup> tested a series of structurally-diverse polymers, containing either peptide or vinyl-derived backbones for ice recrystallization inhibition activity, which is commonly associated with AFGPs. It was revealed that only polymers bearing hydroxyl groups in the side chain could inhibit ice growth. Furthermore, well-defined glycopolymers were shown to have a small, but significant, recrystallization inhibition effect, showing that it may be possible to design antifreeze glycoprotein mimics based upon polymers derived from vinyl monomers.

## 7. Summary and outlook

In summary in this book chapter we reviewed the current advances in biomimetic polymers for chiral and antifreeze applications. The basic principles and potentials of these biomimetic polymers were given for specific examples and as we have present here biomimetic polymers can be very promising materials for controlling chirality and chiral resolution during crystallization. In addition we describe the applications of biomimetic polymers for mimicking the structures, functionality and activity of antifreeze proteins. Although biomimetic polymers can be use for various chiral and antifreeze applications, there is still a knowledge on their molecular mechanism of actions. Study of the complexity of the interactions of biomimetic polymers with chiral crystals and ice surfaces still remains a major challenge in order to develop truly effective biomimetic polymers for those applications. Thanks to new and advanced analytical techniques detailed study molecular and chiral interactions of biomimetic polymers with chiral crystal surfaces is currently feasible. Such research can provide new possibilities for rationally design of various kinds of biomimetic polymers for chiral resolution and antifreeze applications.

We are optimistic that chiral biomimetic polymers will play a critical role in this the development of novel and efficient methods for chiral resolution not necessarily based on crystallization techniques.

In general, the research and use of biomimetic polymers for antifreeze applications is still in its preliminary stages in comparison to their use for chiral applications. Further research

aims to explore mechanism and factors that responsible for the antifreeze activity of biomimetic polymers is still required. Basic issues of fundamental nature, like, interactions of the antifreeze biomimetic polymers with water and their kinetics and energetics of interactions with ice surfaces are still to be addressed. We believe that a deeper understating of molecular mechanism of infractions of antifreeze biomimetic polymers with water and ice surfaces could contribute knowledge in many other fields of research. Moreover, it is obvious that better understating and improved design antifreeze biomimetic polymers is expected to have high potential for many technological applications for instance lengthening shelf life of frozen foods, improving cryosurgery and enhancing preservation of tissues for transplant or transfusion in medicine.

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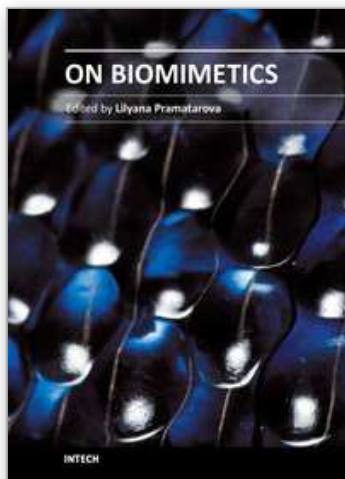
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Bio-mimicry is fundamental idea –How to mimic the Nature™ by various methodologies as well as new ideas or suggestions on the creation of novel materials and functions. This book comprises seven sections on various perspectives of bio-mimicry in our life; Section 1 gives an overview of modeling of biomimetic materials; Section 2 presents a processing and design of biomaterials; Section 3 presents various aspects of design and application of biomimetic polymers and composites are discussed; Section 4 presents a general characterization of biomaterials; Section 5 proposes new examples for biomimetic systems; Section 6 summarizes chapters, concerning cells behavior through mimicry; Section 7 presents various applications of biomimetic materials are presented. Aimed at physicists, chemists and biologists interested in biomineralization, biochemistry, kinetics, solution chemistry. This book is also relevant to engineers and doctors interested in research and construction of biomimetic systems.

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