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Proceedings of the Fifth Royal Society – Unilever Indo-UK Forum in Materials Science and Engineering

SUPRAMOLECULAR AND COLLOIDAL STRUCTURES IN BIOMATERIALS AND BIOSUBSTRATES

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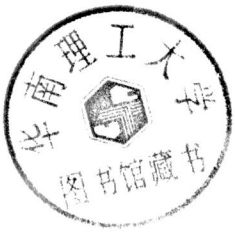

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FOREWORD

This fifth meeting of this series tackled a broad topic in a deliberate attempt to bring together the sciences which are bound to interact in the 21st Century – the established science of physical chemistry and materials with the emerging sciences of molecular biology and cellular organisation. Not only were we attempting a multicultural exchange but also stretching scientists to compare and contrast the approaches and language of these widely separated disciplines.

We sought the similarities, where each branch of the sciences could share with another; the novel control of materials and molecules that the new biology can offer to the physical scientist; and the need for learning new insights from the highly specialised structure of molecules and architecture that biology has evolved.

All of these sciences need new measurement techniques examining dynamics, in real time and non invasively where physicists and biologists already converse and our capabilities have grown immeasurably in recent years.

The object of this unusual event was to open minds and challenge each other about how and why we approach our science in the way we do, and most of all, the change. Such a wide ranging meeting is not intellectually "comfortable", but only then do anomalies emerge and discontinuities become identified. With any luck, this is when innovative approaches are stimulated and new fields of research are conceived.

Thus, we ranged from the systematic design of peptides to elucidate the rules of protein assembly, through the flow induced assembly of colloidal particles to self assembly of hair and skin cells. We saw the elegance of detail we can now detect in crystals, the real organisation of molecules at interfaces, the flow of nutrients in living plants and the power of the modern electron microscope.

What we also learnt was that science is genuinely international in its level of quality. There were many exchanges not only at the sophisticated level of one expert with another, but also at a rudimentary level where the basic inquisitiveness of scientists is exposed when faced with problems they have never previously encountered. For me, it was the latter that showed the experiment was working.

Certainly the conference was a success, if the requests for more are anything to go by. Whether we stimulated breakthroughs in inventive thought only time will tell, but I am sure that I was not the only attendee who travelled home with new insights into how and where new science could be generated and the absolute certainty that its development into technology in the Indian sub Continent will be different and surprising to those of us from the West.

Peter J Lillford, CBE,
Chairman, Organising Committee for the 5th Royal Society-Unilever INDO/UK Forum

PREFACE

The last in a series of five conferences held under the auspices of The Royal Society-Unilever INDO/UK Forum on Materials Science and Engineering, the meeting in Mysore covered biomaterials and biosubstrates, areas of vital importance in technologies concerning food and personal care products. This volume constitutes the proceedings of the meeting, containing both invited and contributed papers.

The programme for the meeting has dwelt mainly on two foci: *i.* measurement of the molecular, supramolecular and colloidal structures of biomaterials and biosubstrates using state-of-the-art techniques, and *ii.* elucidation of the relationship of such structures to the physicochemical, biophysical and mechanical properties through experimentation, theory and simulation. Food materials, skin and hair have featured prominently in the programme.

Nearly half of the 30 papers published in this volume are invited contributions from leading scientists representing major centres of biomaterials research in the UK, the Netherlands, Sweden, Australia and India. They include, among others, Prof E D T Atkins, Prof P Balaram, Prof P Walstra, Prof S B Ross-Murphy, Dr N Chandrakumar, Sir Sam Edwards, FRS, Dr V Prakash, Dr V J Morris, Prof A-M Hermansson and Prof N Appaji Rao. Papers pertaining to focus (*i*) present applications of experimental techniques like x-ray diffraction (XRD), magnetic resonance imaging (MRI), confocal laser scanning microscopy (CLCM), environmental scanning electron microscopy (ESEM), atomic force microscopy (AFM), fluorescence lifetime imaging (FLIM) and fluorescence recovery after photobleaching (FRAP) for measuring interactions and identifying structure at various length scales. Papers corresponding with focus (*ii*) deal with the various phenomena involved in the development, processing, delivery and application/ consumption of personal care and food products, *e. g.,* friction and lubrication, rheology, mechanical strength, gelation, colloidal aggregation/dispersion and film rupture.

The significance of this proceedings volume derives from the high scientific quality of both invited and solicited papers which collectively present a valuable account of up-to-date advances in the field. We believe that the two foci unite in the book in a complementary way, producing a coherent and clear picture of the current level of understanding of how the structure is related to the properties of interest in food and personal care technologies. We are confident that the book will prove to be of appreciable value to most scientists active in the field.

In common with its predecessors, the meeting has fully achieved its essential objective laid down by the Forum, namely, promotion of close contacts between materials research communities working primarily (but not exclusively) in India, UK and European countries by providing suitable platforms in the form of world class conferences, ensuring participation by the UK and the Indian scientists in roughly

equal proportion. It is hoped that the contacts thus forged will be enduring and will prove worthwhile in respect of rapid exchange of new results, seeking reliable sounding boards for new ideas and, possibly, development of new collaborative programmes.

We are grateful to Dr Prem Paul of Unilever Research Port Sunlight and Dr B K Lonsane of CFTRI, Mysore for the invaluable help rendered in the pursuance of various tasks concerning the preparation of this volume.

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CONTENTS

CHAIN-FOLDED apβ-SHEET LAMELLAR STRUCTURES FROM GENETICALLY-ENGINEERED PERIODIC POLYPEPTIDES

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The salient features of the crystal structures and textures of a family of sequence-designed monodisperse periodic polypeptides are presented. In these structures the polypeptide chains fold back and forth in a regular fashion to generate thin (3–6nm) lamellae. In the first instance the repetitive amino acid sequences are of the type $-[(AG)_xEG]-$, with integer x from 3 to 6. The data support an antiparallel (ap) β–sheet structure, and all the structures index on orthorhombic sublattices similar to those reported for *Bombyx mori* silk fibroin. Structures with polar apβ-sheets and γ–turns, stacking with the hydrophobic methyl groups of the alanyl units in contact, are selected by X-ray crystallography to give the best match with experimental data. The results confirm that the folding periodicity is in-phase with the repetitive amino acid sequences so that the glutamic acid (E) residues are confined to the lamellar surface. A series of related polypeptides (keeping $x = 3$) are investigated, in which the glutamic acid unit is replaced successively with: alanine, serine, asparagine, valine, lysine, phenylalanine and tryosine. Analysis of these crystals enables the effect of changing the amino acid side-chain volume on the structure to be investigated. A linear relationship is found between the side-chain volume at the folds and the averageintersheet spacing. The sequence $-[(AG)_3EG(GA)_3EG]-$ was also crystallised and investigated. This structure helps establish which of two structural aspects is dominant: (1) the alanyl-alanyl hydrophobic interactions, or, (2) the γ–turn rather than the β–turn in the fold. The results indicate that the γ–turn is preferred in these chain-folded, stacked pleated-sheet apβ–sheet structures.

1. Introduction

The controlled biosynthetic production of sequence-designed artificial proteins is an emerging area of macromolecular science and technology. In general, we seek to understand the factors governing the relationship between amino acid sequence and spatial architecture of protein molecules. This contribution is a review of recent results on the controlled creation of chain-folded antiparallel (ap) β–sheet crystalline protein structures based on repetitive amino sequences that relate to the silk-like β–proteins.[1-5]

It had been reported by Keith, Lotz and coworkers,[6-9] that sequential polypeptides in the β–conformation could be crystallised in the form of chain-folded lamellae with thickness of the order of 5 nm. In addition, it is generally accepted that adjacent re-entry, chain-folded lamellae is a common crystalline form in linear polymers that are sufficiently flexible to form hairpin-like folds. It is also well-known, from crystallography of globular proteins, that the polypeptide chains can

make sharp folds (turns), with either one or two amide units (two or three amino acid C_α atoms) in the turn; known as β- or γ–turns, respectively.

Some of the perceived advantages creating such chain-folded lamellae would be as follows. (1) A regular chain-folding structure would contain two (β-strand and chain turn) of the three prominent and recognisable protein conformations (the third being the α–helix). Thus, the results would be pertinent to protein structure and folding in general. (2) Most flexible synthetic polymers crystallise by chain-folding, and there is a great deal known about the kinetics and thermodynamics of the folding process. It would be useful to know how subservient proteins are to these general rules of behaviour. (3) The apβ–sheet has established X-ray diffraction and spectroscopic fingerprints, so it should be possible to test for its presence during and after crystallisation. (4) The surfaces could be decorated with useful amino acid to form particular spatial arrangements on the surface; upper and lower surfaces could be decorated differently. (5) Chain-folded lamellae are membrane-like and as such have many possible biological uses. (6) The effect of amino acid sequence on lamellar thickness and surface roughness could be investigated in a controlled manner.

In the first instance, the chain-folded crystal structures from a group of four monodisperse, genetically-engineered polypeptides, represented by the repetitive amino acid sequence: -[$(AG)_xEG$]-, for integer x = 3, 4, 5 and 6, are discussed. The effect of the increasing length of the alanylglycyl (AG) segments is examined. In a separate set of experiments the changes in crystal structure, when the glutamic acid unit (E) is replaced by other amino acids, are investigated. Finally, for poly$(AG)_3EG$, the consequences on the crystal structure when every other $(AG)_3$ segment is reversed will be considered.

2. Choice of Poly$(AG)_XEG$ Sequence

The alanylglycyl (AG) dyads were selected because they are common in many ß-silks and form extended ß-strands, which usually assemble into apß-sheets and stack to form stable crystals.[10,11] Adjacent chains in an apß-sheet, as the notation implies, run in opposite directions, a fundamental ingredient in the crystal design. Thus, it was hoped that chain-folded lamellae could be prepared by crystallising from solution and that the $(AG)_x$ segments would create the straight-stems and the additional EG dyad would lie in the fold. The range of 3 to 6 alanylglycyl dyads (8- to 14-mer repeating sequences) was chosen after consideration of the anticipated thickness of the chain-folded lamellae.

Glutamic acid (E) was incorporated into the repetitive sequences because of its bulk, polar nature, and chemical reactivity. The large size of E relative to A and G should also deter its inclusion into the crystalline lamellar interior on steric grounds: the A-to-A intersheet spacing for polyAG[12] is 0.498 nm, whereas the smallest intersheet distance reported for the ß-form of poly(E) is 0.782 nm,[6] nearly 60%

larger. During crystal growth, interactions with the solvent should also act to segregate the glutamic acid residues to the lamellar surface. The detailed crystallisation procedures and preparation of oriented mats suitable for X-ray diffraction analysis is reported elsewhere.[2]

In order to aid the understanding of these chain-folded polypeptide structures the salient features of the apß-sheet silk structures are first summarised.

3. Antiparallel ß-Sheet Structures found in Silks

These structures consist of sheets composed of relatively extended protein chains with a 2-fold helical conformation. Adjacent, antiparallel chains zip together through linear hydrogen bonds to form the so-called apß-sheet structure. Figure 1 is a computer-drawn model showing a view orthogonal to an apß-sheet with a repetitive alanylglycyl sequence.

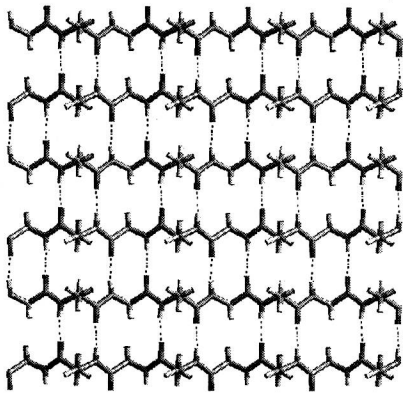


Figure 1. View of a single polar apß-sheet, with a repetitive alanylglycyl sequence, orthogonal to the *ac*-plane.

The repeating distances along the chain axis (*c*) and in the hydrogen bond direction (*a*) are essentially invariant and therefore act as characteristic and diagnostic values for apß-sheet structures. Reported values[11] of *a* and *c* for *Bombyx mori* silk are 0.948 nm and 0.695 nm, respectively. There is a noticeable out of plane puckering of the sheets, as shown in Figure 2. For repetitive alanylglycyl sequence there are two types of apß-sheet. The first arrangement is when the decoration patterns on upper and lower surfaces are different, i.e. the glycyl hydrogen atoms on one surface and the alanyl methyl groups on the other, as shown in Figures 1 & 2a. This may be described as a polar apß-sheet. The second is when the decoration patterns on the

upper and lower surfaces of the apß-sheet are identical, i.e. an equal mixture of glycyl hydrogen atoms and alanyl methyl groups on both surfaces as illustrated in Figure 2b.

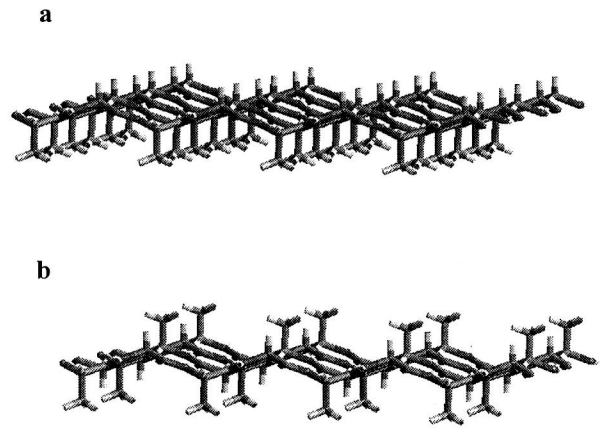
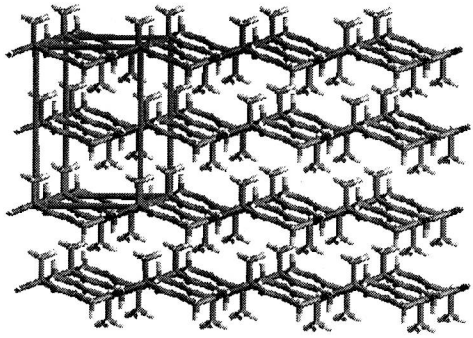


Figure 2. Oblique view of apß-sheets with repetitive alanylglycyl ß-strands; (a) polar apß-sheet and (b) apolar apß-sheet.

This can be described as an apolar apß-sheet. In the full three-dimensional structure the sheets stack with in-phase puckering but usually with alternating or random shears of ca. ± *a*/4 in the *ac* plane. The sheet stacking distance, which is related to the crystallographic *b*-axis, is a function of the size and distribution of the amino acid side groups that decorate the upper and lower apß-sheet surfaces. In the orthorhombic protein apß-sheet structures, where *a, b* and *c* are mutually orthogonal, there are two apß-sheet stacking categories that need to be considered for the benefit of the structural analysis of poly(AG)$_3$EG. Apolar apß-sheets would be expected to stack as shown in Figure 3a, i.e. with equal (*b*/2) spacing. In this structure, the 0*k*0 diffraction signals for *k* odd would be systematically absent. In particular, the 010 would not be observed. On the other hand, polar apß-sheets stacked with like surfaces together as shown in Figure 3b, and as reported for example for polyAG,[12,11] would have the centre sheet in the unit cell displaced from the *b*/2 position and a 010 diffraction signal would be observed; furthermore, its relative intensity would be a convenient indicator of the deviation from equally-spaced apß-sheet stacking in the *b* direction. Thus, these ß-silk-like structures are held together with covalent bonds in the chain (*c*-axis) direction, hydrogen bonds at right-angles (*a*-axis) and van der Waals forces in the third mutually orthogonal *b*-direction.

a



b

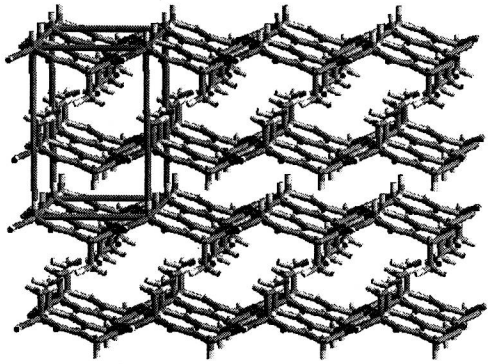


Figure 3. (a) View of stacked apolar apß-sheets; the orthorhombic unit cell is drawn as a box. The 010 diffraction signal would be absent in this structure. (b) View of polar apß-sheets, stacked face to face and illustrating how the hydrophobic methyl groups form layers and displace the centre sheet from the *b*/2 position. Thus in this case, the 010 diffraction signal would be expected to be present.

4. X-ray Diffraction Results

Poly(AG)$_3$EG

The wide angle x-ray diffraction pattern of poly(AG)$_3$EG, taken with the x-ray beam directed parallel to the plane of a oriented mat and with the mat normal horizontal, exhibits discrete Bragg diffraction signals consistent with an oriented crystalline polymer and all the observed diffraction signals index on an orthorhombic unit cell with dimensions a = 0.948nm, b = 1.060nm, and c = 0.695nm. A schematic diagram

showing the indexing of the diffraction signals is shown in Figure 4. A noticeable feature is that the orientation direction is along *a*. Thus, the directionally coincident *a* and *a** axes lie along the meridian line and therefore the *h*00 diffraction signals, the easily seen 200 and 400 in particular, appear as arcs, centred on that meridian. Furthermore the families of 0*k*0 and 00*l* diffraction signals appear on the equator.

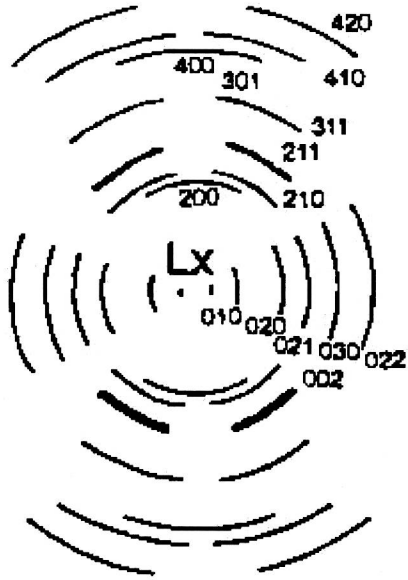


Figure 4. Schematic illustration of the X-ray diffraction pattern from poly(AG)$_3$EG with the beam directed parallel to the plane of an oriented mat: mat normal horizontal. The diffraction signals are indexed and the low-angle (Lx) diffraction signal appears on the equator at 3.6 nm.

Differences in the line broadening of the various diffraction signals, which relate in a consistent way to their assigned Miller indices, are observed. The 200 and 400 diffraction arcs are particularly sharp. This demands long-range correlation along the *a* axis. Diffraction signals with indices of the general *hkl* type are considerably broader than those with indices *hk*0. In particular the 211 diffraction signal is estimated to be tenfold broader than its 210 near-neighbour. Comparison of the line-widths leads to the conclusion that the coherent scattering length in the *c* direction is small; an estimated length of $\approx$4 nm allowing for instrumental line broadening. The relative breadth of the prominent 010 and 020 diffraction signals suggests that the coherent scattering length in the *b* direction is intermediate between those in the *a*

and *c* directions. A low-angle X-ray diffraction peak, at a spacing of 3.6 ± 0.1 nm, is found on the equator.

Poly(AG)$_x$EG for x=4, 5 and 6

The overall features of the x-ray patterns are similar to those of poly(AG)$_3$EG and they all index on orthorhombic unit cells with the same *a* and *c* parameters. There is a continual reduction in the value of *b* within the family as shown in Table 1.

Table 1. Unit cell *b*-values, in nanometers, for the poly(AG)$_x$EG series, with the value[3] for poly(AG) for comparison.

Polypeptide sequence	*b*-value in nanometers
Poly(AG)$_3$EG	1.060
Poly(AG)$_4$EG	1.028
Poly(AG)$_5$EG	0.970
Poly(AG)$_6$EG	0.962
Poly(AG)	0.922

Also, diffraction signals with indices of the type *hkl* sharpen as the length of the repetitive alanylglycyl segment increases. Specifically, the diffraction signal indexed as 211 is approximately twice as sharp in poly(AG)$_4$EG as in poly(AG)$_3$EG, and the relative sharpness increases further through the series, i.e. as x increases. Low-angle arcs, from interlamellar stacking, also occur on the equator but with increasing in spacing (up to 6.2 nm) with increasing x value.

5. Structure of Poly(AG)$_3$EG Crystals

The line broadening of diffraction signals with Miller index $l \neq 0$, the 211 in particular, indicates that there are interruptions to the crystallographic lattice in the *c*-direction, that is, the chain direction, at distances ≤ 4 nm. This is ca. 25 times less than the length of the protein chain. The low-angle signal at a spacing of 3.6 nm, resembles an inter-particle interference function. This diffraction peak can be made to disappear with swelling agents without affecting the underlying crystal structure. This behaviour is reminiscent of inter-lamellar stacking and is similar to that reported for chain-folded lamellar crystals of polyethylene,[13,14] nylons[15,16] and *Chrysopa*[17] insect silk. Adjacent chains in the structure are antiparallel and the straightforward explanation for these results is that the chains in poly(AG)$_3$EG