The Slimy Deadly Weapon of Velvet Worms: an Inspiration for Bio-Compatible Adhesive Materials

Evgenios Stylianidis (s3667308)
Supervisor: Prof. dr. Marleen Kamperman

Zernike Institute for Advanced Materials, University of Groningen

ABSTRACT

Velvet worm’s hunting weapon consists of a sticky slime ejected from animal’s oral papilla which rapidly dries forming a stiff material that entraps the prey. The bio-adhesive secretion of velvet worms presents some peculiar properties that make it a great inspiration for new generation biomimetic polymeric materials. Although initially the slime is ejected in a liquid state, upon mechanical shearing its monomers are supramolecularly self-organised into a fibrillar polymeric material with a high elastic modulus of around 4 GPa, comparable to glassy synthetic polymeric materials. Upon rehydration, the solidified bio-adhesive experiences a phase transition to liquid state, from which new fibers can be formed after stretching. Here, we give an overview of the current understanding on the properties that characterize the secretion slime of velvet worms, creating the starting point for the development of new bioinspired materials. Initially, we investigate the features of the responsible organs and the process of the biosynthesis of the slime. Then, we study the biochemical composition and the structure of the slime, and finally, we summarise all the available information related to the reversible phase transition mechanism of the slime. Finally, we conclude that the natural glue of velvet worms could be the inspiration for new generation bio-compatible polymeric materials. However, a lot of progress in the understanding of the adhesive mechanism should be made to proceed in the bio-mimetic fabrication.

Table of Contents

1. Introduction .......................................................... 1
2. The Bio-Fabrication of the Secretion Slime ................ 3
   2.1 The slime glands features and structure ...................... 3
   2.2 The biosynthesis of the slime .................................. 4
   2.3 The secretion mechanism ....................................... 5
3. Biochemical Composition and Structure of the Adhesive Slime ................................. 6
   3.1 Methods for the compositional characterisation of adhesive secretions proteins ............................................. 6
   3.2 Biochemical Composition of the secreted adhesive slime of velvet worms .......... 8
   3.3 Structure of the secreted slime .................................. 10
4. The Reversible Adhesive and Solidification Mechanism ............................................. 13
   4.1 A non-covalent reversible cross-linking mechanism ............. 13
5. Conclusions and Perspectives ...................................... 14

References ........................................................................... 15

1. Introduction

The ability of nature to develop elegant materials that are well-adapted to their purposes has become an inspiration for researchers in the last two decades for the establishment of a new generation of advanced materials. A wide field of advanced bio-inspired materials is grown in the last few years, based on the strategies, the complexity and the mechanisms that biological organisms use for the production of biomaterials useful for their survival. Marine and terrestrial animals have been studied in recent years, such as sandcastle worms (Phragmatopoma californica)¹, barnacles², mussels³, spiders⁴, and geckos⁵, for the interesting properties of the protein-aqueous bio-adhesive materials that they produce not only for settlement but also for their defence, nest construction, mobility or prey capture.

Recent investigations have focused on the adhesive slime secreted from a rarely-found animal, the velvet worm. The secreted liquids ejected from these species show a liquid-to-solid phase transition after its extraction, in a reversible manner under
environmentally friendly conditions. Under tension, the ejected liquid forms a stiff fibrous material with an elastic modulus of around 4 GPa, comparable to the synthetic man-made Nylon\textsuperscript{6}, and after dissolution in water the fibrilic material dissociates in its building blocks.\textsuperscript{6} The interesting properties of this secreted material, in combination with the out-of-the-body phase transition of the slime, has attracted the interest for a new environmentally friendly bio-inspired material development. However, the natural mechanism that describes the properties of velvet worms slime is still unclear and dispersed and limited amount of information is available.

**Onychophoran (Velvet Worm)**

Members of the phylum Onychophorans, velvet worms are carnivorous, soft-bodied terrestrial invertebrates.\textsuperscript{7} These rarely found animals inhabit temperate and tropical moist forests in the Southern Hemisphere and around the Equator, and they live mostly in rotting wood, leaf litter or soil, while they are more rarely found in caves’ moist environments.\textsuperscript{8,9} The two extanted families of velvet worms are Peripatidae and Peripatopsidae. Hitherto, 191 valid velvet worm species have been described, namely 76 *peripatids* from Central and South America, Western Africa and South-East Asia, and 115 *peripatopsids* from Chile, South Africa and Australia.\textsuperscript{10-12}

Velvet worms have a uniform semi-cylindrical body, with length ranging from 10 mm to 15 cm. However, giant species of Onychophoran have found with length around 20 cm.\textsuperscript{13} Known species have 13 and 43 pairs of laterally inserted uniform stubby limbs, which are referred to as lobopods.\textsuperscript{14} Each of their limbs terminates in a couple of claws, that also gives the name of the Onychophorans.\textsuperscript{15} Unlike arthropods, velvet worms do not have an exoskeleton, hence, their limbs do not need joints to facilitate mobility. Their body is covered with a velvety skin, full of large and soft papillae, which acts as a water repellent.\textsuperscript{16} On their head is a pair of soft and mobile antennae, serving as sensing organs. The ventrally located mouth is equipped with a set of sclerotised jaws, and is flanked by two slime-extracting papillae, which are the source for the secretion of the importantly interesting adhesive slime.\textsuperscript{17}

One of most unusual features of velvet worms is their hunting and feeding habits. Onychophorans are in general slow-moving invertebrates, so they are ambush predators. They hunt during the night and they detect and approach their victims by their sensitive antennae and skin. After choosing the suitable victim, they immobilise their prey by ejecting two streams of a sticky liquid via a pair of slime papillae, Fig. 1B. Immediately after release, the slime hardens and the prey is entangled in a net of sticky threads, Fig. 1C,D. Then, the velvet worm approaches its victim, and they consume
their prey using its jaws, strong digestive enzymes and a sucking pharynx, Fig. 1E.8,17

The secreted sticky glue of Onychophorans possesses a wide range of interesting properties. Nowadays, efforts are made to gain an understanding of the physical and chemical properties of this adhesive secretion. In the present work, we aim to review all the available spread information related to the sticky slime of velvet worms, in order to reach a good level of understanding of this complex biological material and create the starting point for the development of new synthetic bio-compatible materials. Initially, we discuss the morphology and features of the velvet worm secretion glands, which are the origin of synthesis for the adhesive liquid. Then, we summarise all the information based on the biochemical composition of the ejected slime, referring simultaneously to the most used techniques for biochemical analysis. Continuously, we examine the liquid-to-solid phase transition mechanism of the secreted material, and finally, we conclude with the perspectives and promising applications.

2. The Bio-Fabrication of the Secretion Slime

The unique mechanism of prey capture in velvet worms is based on the existence of prominent slime glands, which produce and store the secreted liquid. In this section, we examine the main features of those responsible organs for the formation of the slime. Following, we investigate the bio-synthesis process which is followed inside the secreted glands, and finally, we examine the mechanism of ejection of the slime.

2.1 The slime glands structure and features

The slime of Onychophorans is formed into long paired structures which are located on the main part of their body (central sinus) on each side of the gut, the slime glands. These organs are binded with the regular intervals via connective tissues, as depicted in Fig. 2A,C, which contain tracheal tubes and densely packed longitudinal muscle fibres and nuclei. Each slime gland is divided into two major parts: an anterior syringe-like reservoir in which the slime is produced and stored and is discharged by ducts in the oral papilla, and a posterior brush-like glandular portion associated with numerous endpieces.19

The glandular epithelium is constituted by prismatic cells that line the cavity of the endpieces. These cells have nuclei larger than those of the other tissues, as shown in Fig. 3A-C and they are densely packed with tubules of rough endoplasmic reticulum (RER) and Golgi vesicles.15,17,19 The prismatic cells in the slime gland epithelium are responsible for the biosynthesis of the slime via the regulated secretory pathway and for the presence of water and proteins in the biochemical composition of the slime, as they are well adapted for rapid and continuous protein synthesis.20

![Figure 2: Optical microscope images of the attachment motif of Onychophoran slime glands. (A) Strand of connective tissue connecting the secretory duct to the gut in Peripatopsidae species. With arrows are depicted the whitish tracheal tubes within the strands. (B) Strand of connective tissue attaching the reservoir to the inner surface of the body in a Peripatopsidae species. (C) Lateral gut surface showing a laterial bar of connective tissue filled with white tracheal tubes (arrows heads) in a Peripatidae species. (D) Secretory duct attached to the connective tissue in a Peripatidae species. cs: connective strand, du: secretory duct, ep: endpieces, in: intestine, re: reservoir. Scale bars: (A) and (C) 50 μm, (B) 150 μm and (D) 25 μm. Images are reproduced from Baer and Mayer19](image-url)
difference of the two species is the arrangement of the endpieces along the secretory duct of the gland. In the Peripatopsidae species, the endpieces project into the body cavity without any obvious regular pattern. In contrast, the Peripatidae species show endpieces which are condensed in regularly spaced rosettes along the secretory duct. Each rosette contains two pairs of fused endpieces that appear as two bifurcating structures.

Optical microscopy studies performed by Concha et al. on the anatomy of the secretion system have shown a syringe-like geometry of the secretion organ. As shown in Fig. 3D,E, the large elongated reservoir continues with a narrow duct that ends at the oral papilla. The capacity of the reservoir in secreted slime reaches up to 11 % of the entire body mass. A well-organised system of helical and circular fibres makes up the wall of the reservoir, while in most cases, Baer and Mayer observed a lack of complex organisation of musculature in the glandular region.

2.2 The biosynthesis of the slime

The presence of prismatic cells in the glandular epithelium of velvet worms suggests the bio-formation of the slime from the so-called regulated secretory pathway that drives the formation, packaging and ejection of the adhesive proteins via the adhesive organs. As shown in Fig. 4, initially, genes encoding adhesive proteins are transferred to the cytosol through matured mRNAs, in where they are translated in the ribosomes. The rough endoplasmic reticulum (RER) then captures secreted proteins from the cytosol as they are being synthesised. The newly formed proteins are released into the lumen of the RER, and they are then transferred from this compartment to the Golgi apparatus. Nextly, selected proteins in the trans-Golgi network are diverted into secretory vesicles, where the proteins are concentrated and stored until an extracellular signal stimulates their secretion.

During the regulated secretory pathway, the slime proteins are ejected into the reservoir cavity in secretory vesicles. However, in the case of velvet worms, there are disagreements between researchers, while some support the observations of swelling and bursting of the vesicles, while some others support the idea of apocrine secretion. In a transmission electron imaging investigation of Ruhberg and Storch in freshly secreted droplets, membrane residues and micellar particles were observed, while such particles were absent in droplets 12 minutes after secretion while they had also lost their adhesiveness. Indeed, a structural and compositional analysis of the secreted slime confirmed that the ejected slime constitutes a suspension of spherical monodisperse nanoglobules.
2.3 The secretion mechanism

The well-organised muscular arrangements in the walls of the reservoir, which provide the robustness and flexibility needed for the reservoir cavity, in combination with the observed peristaltic contractions of the reservoir, were suggested to involve in the mechanism of slime ejection. However, these contractions alone might not be sufficient for the powerful secretion mechanism of the slime. Moreover, a proposed squirting mechanism based on whole-body contractions was later rejected due to the observed non-synchronized secretion from the two papilla of a representative animal. Despite the failure to describe the ejection mechanism, it is suggested that the contractile behaviour of the reservoir’s walls, together with the valve-like structure at the junction between the secretory duct and the reservoir might prevent the slime from flowing back into the secretory portion of the gland during ejection process.

Observations of swinging movements of the oral papilla during squirting led Morera-Brenes and Monge-Najera to also suggest that muscular actions are the driving force for the ejection of fluid streams. More specifically, those observations led them propose that muscular actions force the oral papilla to swing and secret powerfully the adhesive liquid. However, the role of muscular contractions was later put into question by an analytical study presented in the fast oscillatory motion of the oral papillae. The oscillations of the oral papillae during squirting were observed to be faster than any other known muscular motion of the worm. As a result, the driving of the oscillations of the papilla by any muscular contraction is conceptually difficult.

The work of Concha et al. on the oscillatory motion of
the nozzle-like papilla of velvet worms provides evidence that the secretion mechanism is driven by a simple physical elastohydrodynamical instability, that arises from the competition of fluid inertia and the papilla elastic resistance. As mentioned, in microfluidic systems, in where sizes are small, inertial effects are important when the velocities of the fluid are sufficiently large. For fluids moving with a large speed in flexible nanoscopic systems, centrifugal and Coriolis forces can be developed and make the system unstable when the speed of liquid is above a critical speed of motion \( V_c \), that depends on boundary condition. In the case of velvet worms, the contractions of the reservoir, together with the geometric amplifier shape of the reservoir-papilla system (Fig. 3D,E) can lead to the acceleration of the fluid during squirting, reaching velocities large enough to generate instability.\(^{21}\) Indeed, for a *Peripatus solorzanoi* specimen the group measured jet speed of \( V \sim 3.2 - 5.0 \text{ m s}^{-1} \) and calculated a Reynolds number (the ratio of inertial to viscous forces, it characterizes the fluid inertia) \( Re \sim 2700.\(^{21}\) The critical speed for the specimen’s oral papilla was calculated at \( V_c \sim 3 \text{ m s}^{-1}.\(^{21}\) Consequently, due to that simple physical instability, the oral papilla can become unstable squirting the powerful adhesive slime without the presence of muscular contractions.\(^{21}\)

3. Biochemical Composition and Structure of the Secreted Slime

Biological adhesive secretions can be notably complex and contain a large variety of components with different properties and interactions. Natural adhesives are mainly composed of proteins, which allows their compositional characterisation via molecular methods. In this section, we try initially to summarise the experimental strategies that are primarily used for the compositional characterisation of bioadhesive slimes. Furthermore, we summarise the biochemical compositional analysis of the secreted slime of velvet worms, accompanied finally by the structural characterization of the Onychophoran adhesive material.

3.1 Methods for the compositional characterisation of adhesive secretions proteins

Santos et al. have presented an extensive review of experimental methods for the characterisation of bio-adhesives proteins.\(^{23}\) Based on the adhesive-cell secretory pathway biosynthesis, two different approaches are mainly used for the compositional characterization of bio-adhesive materials. The two methods consist of a molecular biology approach and a protein chemistry strategy, and they are schematized in Fig. 5.

3.1.1 Molecular Biology Strategies

Molecular biology techniques involve the construction of complete primary sequences from the cell nucleotidic information (DNA or mRNA). This information can be extracted from the adhesive organs, in order to get access to the genes and therefore to the adhesion proteins contained in adhesive materials. However, the identification of adhesion-related genes and mRNAs can be problematic, due to the limited or completely unavailable amount of information, sequence data or genomic database for many organisms.\(^{23}\)

The construction of a complementary DNA (cDNA) library and sequencing was used until recently for the generation of expressed sequence tags.\(^{24}\) A cDNA library is a combination of cloned cDNA fragments which are inserted into a collection of host cells, which together constitute some portion of the transcriptome of the organism. The main steps of this process are (i) the isolation of mRNA, (ii) the reconstruction of cDNA via reverse transcription of mRNA and (iii) the ligation of cDNA fragments into bacterial plasmids. For further identification of adhesive proteins, bioinformatics tools can be used after the transcriptome construction. In addition, random sequencing, as well as polymerase chain reaction (PCR), were combinedly used to screen the secretion gland cDNA library of spider webs.\(^{25}\)

Nowadays, direct access to mRNA information is achieved by transcriptome sequencing due to the development of the next-generation sequencing (NGS).\(^{23,26,27}\) This process involves several steps such as: (i) isolation of mRNA, (ii) fragmentation of mRNA, (iii) construction of a sequencing library with adapters on both ends, (iv) paired-end sequencing, and (v) bioinformatic assembly of paired-end reads. In the case when a small amount of adhesive material can be collected or when the protein information is hindered due to insolubility, a differential RNA-sequence approach can be applied for the generation of the adhesive-related gene list. During this procedure, a
tissue with and a tissue without adhesive cells are sequenced using short reads, which are then mapped to the existing transcriptome to identify differentially expressed transcripts. This approach has been used in investigations of the marine flat-worm Macrostomum lignano\textsuperscript{28} and of the cement proteins of barnacles\textsuperscript{29}.

After the characterisation of an adhesive protein, molecular biology techniques such as reverse transcription PCR (RT-PCR) can also be used for the full-length sequence of proteins without the need to sequence a high number of transcripts. In addition, repeated amino acid sequences of the known adhesive proteins can be used for the identification of the homologous proteins. The final step for the full characterisation of adhesive proteins consists in silico analyses via bioinformatics tools. Predictions about the physicochemical properties of the adheres materials can be obtained based on the complete primary sequences. Indeed, a biochemical investigation of the adhesive material of velvet worms reported by Haritos et al. was based on the amino acid composition of the proteins of the secreted slime of velvet worms.\textsuperscript{30} Using the Rapid Automatic Detection and Alignment of Peptides tool (RADAR), as we will see below, they identified tandem repeats of consecutive charged residues with short hydrophobic regions in the velvet worms proteins Er_P1 and Er_P3.\textsuperscript{30}

Finally, available tools are also able to predict sites for post-translational modification (PTM) such as glycosylation, hydroxylation and phosphorylation in the primary sequence.\textsuperscript{23} Such a process is also mentioned in the investigations of Haritos et al. for the adhesive proteins of velvet worms.\textsuperscript{30}

3.1.2 Protein Chemistry Techniques

Protein chemistry tools give a direct route to get polypeptidic sequences and post-translation modifications (PTM) information from the ejected material or their precursors’ cells in the responsible organ. In some organisms, the isolation of the adhesive protein can be difficult, due to the insolubility of the adhesive material, or due to the presence of strong denaturing or reducing conditions.\textsuperscript{31–34} To overcome the problem of proteins isolation, protein extraction can be performed through the adhesive glands, in where soluble adhesive protein can be found.\textsuperscript{23} However, in the case of velvet worms, isolation of adhesive proteins can be performed directly from the secreted material, a consequence of its soluble nature.\textsuperscript{18}

When adhesive proteins are dissolved, different purification methods can be followed for the separation of proteins. Purification methods are based on various characteristics of the proteins: (i) size, by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE), (ii) size and isoelectric point, by two dimensional PAGE, (iii) physicochemical properties, by precipitation with ammonium sulphate or acetone, and (iv) binding affinity, by using different types of chromatography.\textsuperscript{23} Subsequently, peptide sequencing follows for the identification of adhesive proteins. The two main techniques that are used to obtain peptide fragments sequences are Edman degradation and mass spectroscopy (MS). Edman degradation implies the labelling of N-terminal amino acids which are then cleaved and can be identified, without the disruption of the peptide bonds between other amino acid residues. A cyclic repetition of that process can then construct the sequence of the peptide.\textsuperscript{23}

Mass spectroscopy technique, in principle, ionizes biomolecules and their mass is measured from specific trajectories in a vacuum system. Two different approaches are developed hitherto for the identification of proteins via MS: a top-down proteomics, in which large protein fragments occur gas-phase fragmentation, and bottom-up proteomics, in which complex protein mixtures are initially subjugated to proteolytic cleavage, and then peptide products are analysed by MS. During MS, peptides resulted by enzymatically digested adhesive proteins are usually sequenced. The two most well-used techniques to construct protein sequences via MS are peptide mass fingerprinting (PMF) and tandem mass spectrometry (tandem MS or MS/MS).\textsuperscript{23}

Overall, the two different developed techniques for the identification of adhesion-related proteins, protein chemistry and molecular biology, offer distinct advantages due to their different approach, and they can be well used independently. However, a combination of the two techniques, a dual proteomic and transcriptomic approach, it is the best way to achieve the identification of novel adhesive protein and the construction of complete proteinic sequences, as the two techniques are complementary.
3.2 Biochemical composition of the secreted adhesive slime of velvet worms

The Onychophorans slime is mainly composed of water and proteins, as elementary investigations observed.\textsuperscript{16,35} This observation is in line with the glandular epithelium of the slime gland, as it is composed of cells that are well adapted for rapid and continuous protein synthesis.\textsuperscript{20} The amount of water in the slime differs slightly in different species, as it was observed to occupy around 84% in \textit{Peripatopsis moseleyi}\textsuperscript{36} and up to 90% in \textit{Euperipatoides kanangrensis}\textsuperscript{18}. An analytical investigation of Benkendorff et al. on the biochemical secretion composition of the species \textit{E. kanangrensis} showed that the slime is composed of water, protein, sugar, lipid and surfactant nonylphenol.\textsuperscript{18} This composition is also supported for the species \textit{Euperipatus hilkae}, as the researchers observed a proteinaceous-based slime that also contains carbohydrates.\textsuperscript{37}

Sugar composes around 1.3% of the dry weight of the slime, with the main representative to be galactosamine (N-acetylgalactosamine, GalNAc).\textsuperscript{18}
Galactosamine appears to be the only sugar in high molecular weight proteins, and it was observed to be O-linked to the protein by its single residue GalNAc. After an experiment of β-elimination of the GalNAc, Benkerdorff et al. found that Thr is the predominant glycosylated amino acid. The GalNAc residue was also observed to be the dominant residue in the sugar composition of other bioadhesive materials, such as the orb spider web and the silkworm Bombyx mori. Moreover, the glycosylated Thr was observed to be present in the Y position of the Gly-X-Y repeat in the cuticle collagen of the vestimentiferan worm Riftia pachyptila. These observations made the group of Benkerdorff et al. to propose that the glycosylation observed in the secreted slime of velvet worms cannot describe the adhesive behaviour of the slime, but it is more reasonably correlated with the structure of the extensible fibre-like thread.

In general, the presence of sugars in aqueous proteinic solutions was shown to increase the stabilization of proteinic macromolecules in solution. Furthermore, for systems that undergo self-assembly it was observed that the presence of sugars facilitates the self-assembled state, since the presence of contacts between protein molecules via sugar moieties decreases the surface area of the protein and hence their chemical potential charge per monomeric unit. During dehydration, sugars could also replace hydrogen bonds between proteins and water stabilizing the solid material. With that, we propose that sugars may have a mutual role in the adhesive slime of velvet worms, by stabilizing the slime in liquid state, and reinforce the self-assembly fiber formation during the dehydration of the slime. However, this is not yet clear and an analysis in the role of sugars in such bioadhesive systems will help in the biomimetic strategies.

The presence of lipids in the slime of velvet worms was also identified. Observed lipid enclosures and micellular particles also indicate the presence of lipids in the composition of the slime. Furthermore, the presence of a lipid suspension, in where proteinic nanoglobules were dispersed, was observed in ejected slime. The role of lipid was investigated for many natural adhesives, and was mainly found that lipids main function is to facilitate the attachment of the adhesive material in aqueous surfaces and improve the adhesion of proteins. Moreover, the surfactant nonylphenol was also detected in the secretion of velvet worms, and this is the first reported presence of nonylphenol in a natural source. Benkendorff et al. suggested that lipid and nonylphenol may function as an anti-adhesive substance for the slime glue to the walls of the reservoir and the slime gland, or may help in the ejection mechanism due to their fluidity. In addition, they hypothesised that nonylphenol, due to its non-volatile nature, may contribute to the retardation of drying of the ejected slime and maintain the wetting of the waxy insect skin for better prey capture. Finally, it was suggested that lipids in the velvet worm slime may prevent the self-assemble of proteins before the application of mechanical tension.

Proteins contribute the 55 ± 10% of the dry weight of the slime, and consequently, they are the major molecular component of the secretion slime of velvet worms, and responsible for its properties. Proteins are spread in a wide range of molecular weights between 8 to 1300 kDa, as was observed after SDS polyacrylamide gel electrophoresis (SDS-PAGE). Several biochemical investigations of the secreted slime proposed the separation of proteins in three main size groups; proteins with low molecular weight between 8 and 25 kDa, mid-sized proteins with molecular weight between 55 and 110 kDa, and large size proteins with molecular weight between 350 and 1300 kDa. Indeed, an analysis using cDNA library confirmed the size variability of proteins in the slime but has also shown that high-molecular-weight proteins compose the majority of slime. Furthermore, a slime protein profiling study in several species of velvet worm has indicated that the composition of proteins in different species of Onychophoran differs mainly in the low molecular weight proteins, while the most common characteristics were observed in high molecular weight proteins. With this observation, we hypothesize that this is also an indication that the high molecular weight proteins might be responsible for the out-of-the-body specific properties of the slime of velvet worms, as the slime of different species was observed to have the same properties.

The analysis of Haritos et al. proposed the separation of proteins of the Onychophoran slime in three major groups: proline-rich proteins, lectins and small peptides. The class with the high abundance in proline, proteins with molecular weight greater than 200 kDa, is indicated to be the most represented in the slime gland, and is agreed with the investigation of
Benkendorff et al., as the amino acid sequences contain residues rich in proline, as it can be seen in Table I. The primary members of the high proline amount (>20%) proteins identified in the study of Haritos et al. are: Er_P1 (230/350 kDa), Er_P2a and Er_P2b (80 kDa), Er_P3 (17.7 kDa). Those proteins were proposed to be responsible for the gelation of the secreted slime via mechanisms involving covalent, ionic and/or hydrophobic bonds and they are discussed in the next section. The low molecular weight proteins identified in the slime gland cDNA library, show high homology to protease inhibitors and carbohydrate-binding which are assumed to serve as antimicrobial agents within the slime glands.

Table I: Amino acid frequencies [mol %] for two different species of Onychophoran velvet worms and the amino acid mean frequencies for typical identified globular proteins. Amino acid main characteristics are also presented.

| Amino Acid | Physical Property | Typical Globular protein data | E. rowelli (Er_P1) | E. kanangrensis (high MW)
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Asx (B)</td>
<td>Polar acidic</td>
<td>10.4</td>
<td>13.9</td>
<td>12.2</td>
</tr>
<tr>
<td>His (H)</td>
<td>Polar basic</td>
<td>2.3</td>
<td>3.4</td>
<td>2.5</td>
</tr>
<tr>
<td>Lys (K)</td>
<td>Polar basic</td>
<td>6.1</td>
<td>12.3</td>
<td>8.9</td>
</tr>
<tr>
<td>Arg (R)</td>
<td>Polar basic</td>
<td>4.6</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>Gln (Z)</td>
<td>Polar neutral</td>
<td>9.7</td>
<td>6.3</td>
<td>10.9</td>
</tr>
<tr>
<td>Ser (S)</td>
<td>Polar neutral</td>
<td>6.3</td>
<td>2.9</td>
<td>3.2</td>
</tr>
<tr>
<td>Thr (T)</td>
<td>Polar neutral</td>
<td>6.2</td>
<td>5.5</td>
<td>6.2</td>
</tr>
<tr>
<td>Hyp (O)</td>
<td>Polar neutral</td>
<td>0</td>
<td>20</td>
<td>3.7</td>
</tr>
<tr>
<td>Pro (P)</td>
<td>Polar neutral</td>
<td>4.6</td>
<td>12.1</td>
<td></td>
</tr>
<tr>
<td>Ala (A)</td>
<td>Polar neutral</td>
<td>8.2</td>
<td>1</td>
<td>1.7</td>
</tr>
<tr>
<td>Gly (G)</td>
<td>Polar neutral</td>
<td>8.0</td>
<td>6.4</td>
<td>16.1</td>
</tr>
<tr>
<td>Val (V)</td>
<td>Hydrophobic</td>
<td>7</td>
<td>6</td>
<td>4.7</td>
</tr>
<tr>
<td>Tyr (F)</td>
<td>Hydrophobic</td>
<td>3.6</td>
<td>3.9</td>
<td>3.3</td>
</tr>
<tr>
<td>Met (M)</td>
<td>Hydrophobic</td>
<td>2</td>
<td>0</td>
<td>0.5</td>
</tr>
<tr>
<td>Ile (I)</td>
<td>Hydrophobic</td>
<td>5.4</td>
<td>3.8</td>
<td>3.4</td>
</tr>
<tr>
<td>Leu (L)</td>
<td>Hydrophobic</td>
<td>8.4</td>
<td>3.2</td>
<td>2.4</td>
</tr>
<tr>
<td>Phe (F)</td>
<td>Hydrophobic aromatic</td>
<td>4.0</td>
<td>4.1</td>
<td>3.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th><strong>Amino Acid frequencies by category of physical properties [mol %]</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Charged Residues</td>
<td>23.4</td>
</tr>
<tr>
<td>Hydrophobic Residues</td>
<td>30.4</td>
</tr>
<tr>
<td>Proline</td>
<td>4.6</td>
</tr>
</tbody>
</table>

Values are mol %. Typical globular protein data are reproduced from Peter Tompa and represent the mean values of amino acid composition for identified globular proteins.

In Table I, the amino acid composition of the dominant proteins of two different Onychophoran species are presented, accompanied by the mean values of the typical identified globular proteins, taken from a review of Peter Tompa. As one can see, the slime compositions of the two investigated velvet worm species contained a high amount of both positively and negatively charged fragments, and proline residues in comparison to the average globular protein data, while hydrophobic residues performed low quantities in the slime of both velvet worm species.

An analytical investigation on the crude slime of the E. rowelli species of Onychophoran was done by Baer et al. in order to investigate the presence and the role of ionic fragments in the proteinic sequences of the slime. Phosphorylated proteins and a high abundance of divalent metals such as calcium and magnesium have been identified in the crude slime composition. The presence of phosphorous in the solid slime was also identified in the Onychophoran species Epiperipatus hilae using energy-dispersive x-ray spectroscopy. The presence of phosphorous and transition metals in the slime composition could be an evidence for specific supramolecular ionic bridging between the high molecular weight proteins to form globular and fibrous structures, as further discussed below.

3.3 Structure of the secreted slime

Investigations with Dynamic Light Scattering (DLS) and Atomic Force Microscopy (AFM) in the crude slime of velvet worm have shown that the slime initially constitutes an adhesive lipid-aqueous liquid, with monodisperse particles with a hydrodynamic radius of 75.8 ± 0.6 nm distributed in it, as shown in Fig. 6. Those monodispersed particles are mainly composed of proteins distributed in lipid suspension globules.

Indeed, velvet worm slime is ejected from the oral papilla in the as-described liquid state. However, immediately after release, the liquid slime material is observed to dry, lose its adhesiveness and form a very stiff material in the form of small translucent fiber-like threads (diameter 0.02 mm), each holding regularly aligned droplets, as shown in Fig. 1H. Atomic Force Microscopy (AFM) and Transmission Electron Microscopy (TEM) images have indeed proved the presence of nanoglobules which self-assemble after the application of mechanical shear forming fibre-like structures, and that in the presence of water the material changes to a suspension with a higher density of globular particles connected by a fibre-like material. An analysis with Raman
confocal imaging on the thread-droplet interface has shown that lipids are more abundant in the aligned-to-threads droplets, and the fibrillic core is mainly composed of proteins\textsuperscript{6}. A mechanical study of the dry thread has shown an elastic modulus of around 4 GPa, comparable to silkworm and Nylon\textsuperscript{®}, and a maximum stress and strain of 101.9 ± 20.1 MPa and 3.5 ± 1.2\%, respectively.\textsuperscript{6,16}

In Fig. 7a, optical and confocal images show the particulate texture of the composed from nanoglobules crude slime, and the formation of a fibrous core region surrounded by a particulate coating after the application of mechanical drawing. In Fig. 7b, a scheme of a proposed model for the formation of the fibrous material is presented.
For the understanding of the self-assembly mechanism of the nanoglobules after mechanical shearing, it is crucial to investigate the structure of the proteins which are contained into the nanoglobules. The structural composition of proteins in the slime is not yet clear, as contrasting opinions have been expressed. Several studies have proposed that Onychophorans slime is composed of highly structured fibrous proteins such as silks or collagen, \textsuperscript{48,18} while structural predictions show intrinsically disorder proteins.\textsuperscript{30}

Benkendorff et al. studies on \textit{E. kanengrensis} species have proposed that Onychophorans produced slime with collagen-like structural fibres, as a result of the high abundance in proline, the moderate amount of glycine and a small fraction of hydroxyproline in the large proteins of the slime.\textsuperscript{18} Furthermore, they suggested that the small proteins were responsible for the adhesive fluid that coats the capture fibres.\textsuperscript{18} In addition, the same investigation proposed that the low amount of Ala amino acid in their observations was an indication of the absence of Ala-rich fibroin-like structures, which is present in mussels collagen.\textsuperscript{18,49} Reinforcing the presence of ordered proteins, Fourier Transform - Infrared Spectroscopy (FT-IR) measurements on \textit{Euperipatetus hilkae} species have also indicated the presence of amyloid \(\beta\)-sheet fibrillar protein structures.\textsuperscript{37}

On the other hand, structural predictions of the dominant proteins of the \textit{E. rowelli} species slime (\textit{Er\_P1}, \textit{Er\_P2}) have shown that the proteins present disordered structures with no \(\alpha\)-helix or \(\beta\)-sheet formation, and low glycosylation.\textsuperscript{30} As shown in Fig. 8, their predictions for \textit{Er\_P1} suggested long imperfect tandem repeated units that cover most of the sequence. The predictions for the structure of the \textit{Er\_P2} protein have also shown an extended disordered central region.\textsuperscript{30} As a result, Haritos et al. supported that the proteins of the Onychophoran slime secretion compose a unique set of highly unstructured disorder proteins.\textsuperscript{30} This indication was also convinced from structural characterization analysis on a sample of dried slime; using FT-IR and Wide-Angle X-ray Scattering (WAXS), lack of long-range ordering on the \textit{E. rowelli} slime was observed\textsuperscript{10}, while FT-IR measurements on \textit{Macroperipatus geagy} slime by Jerez-Jaimes and Bernal-Perez indicated a strong amide-I absorbance maximum at 1640 cm\(^{-1}\).\textsuperscript{51} which was proposed to present unstructured protein.\textsuperscript{51}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure8.png}
\caption{Structural predictions of the amino acid sequence of the dominant proline-rich protein \textit{Er\_P1} of \textit{E. rowelli}: shows aligned tandem repeated regions and conservation of sequence and character within the sequence. Residue colours: red, acidic D,E; blue, basic K, R, H; yellow, hydrophobic V, L, F, Y; green, small and polar N, T, P, G, A, Q, glutamine; purple, low frequency C, w between repeats. Conserved proline residues are indicated (\(*\)) below the aligned sequences. Coloured bars represent the average character of the segment at neutral pH: red, negatively charged; blue, positively charged; yellow, hydrophobic. Image reproduced from Haritos et al.\textsuperscript{30}}
\end{figure}

The contrary observations of the structure of the slime proteins in the nanoglobules for different Onychophoran species are not yet clarified if they arise because of phylum differences\textsuperscript{15}, or because the proteins had dried during the measurement, so they would have already formed secondary and tertiary structures during solidification.\textsuperscript{52} We believe that more sensitive and carefully investigations should be done to identify the real structure of the proteins, as it is crucial for the understanding of the mechanism that drives the solidification of the velvet worm slime. As this process occurs out of the body and seems to arise after the application of mechanical force, and not because of changes in the proteins’ environmental composition, the clarification if the proteins are already structured
inside the glands' cavity before the extraction or not would give us a better view in order to explain the phase transition mechanism. Overall, the prevailing view in proteinic structure between researchers is that the proteins compose disordered structures, mostly in the shape of the predicted tandem repeated units of highly charged zwitter-ionic macromolecules.30

4. The Reversible Adhesive and Solidification Mechanism

As it was already described, the slime of velvet worms consists a liquid-like material at rest, but when disturbed by mechanical forces it is dehydrated forming stiff adhesive threads.5,35–37 By washing the solidified fibres with distilled water, it was observed that the material was completely diluted after 8 hours, and a highly concentrated proteinic solution could also regenerate new fibers by new mechanical drawing.6,37 Herein, we will try to summarise the available information and ideas on the out-of-the-body mechanism that drives the reversible fibre formation of velvet worms slime.

4.1 A non-covalent reversible cross-linking mechanism

The dynamical fiber formation due to dehydration of the slime induced by mechanical shearing is an indication of non-covalent interactions between the building blocks that compose the slime.6 The most crucial point of the discussion is to understand what changes during the mechanical shearing of the slime and what interactions are developed resulting in a dry solid material. In general, natural proteinaceous strong gels and adhesives are composed of dilute polymer networks that take a somehow semi-liquid form.53 The two major mechanisms for the formation of this polymer network are the entanglement and a cross-linking mechanism.53 The mechanism of entanglement requires the presence of large (>100 kDa) heavily glycosylated proteins.54 On the other hand, cross-linking mechanism requires interactions between residues that are relatively long lived and depends on the number and the strength of those interactions.53

According to Haritos et al., entanglement is not favourable to be the basic mechanism for the build of the adhesive fibres from velvet worms slime, as the dominant proteins of the glue are only slightly glycosylated and they are present as viscous liquids in the glands.30 However, this explanation is not sufficient because although the presence of sugar moieties in the slime composition is limited, the size of the dominant proteins of the slime is large enough to form entangle networks.

Nevertheless, based on the abovementioned prediction that the proteins of velvet worms form highly charged zwitter-ionic macromolecules30, a model of cross-linking supramolecular interactions between the proteins fragments has proposed by Haritos et al.30 to explain the phase transition of the velvet worm slime.30 For the stability of the hydrated liquid state of the crude slime when at rest, Haritos et al. proposed that the highly hydrated disordered proteins, which are the dominant component of the slime, fail to form secondary and tertiary structures in long-range.30 As hypothesised, this failure stems from the repulsive interactions between charged amino acids residues and the low abundance in hydrophobic fragments.30 The material can be characterized in that phase as an amorphous polymer solution.30 During the mechanical disturbance of the slime system by the prey, adjacent proteins come in such proximity so as ionic and hydrophobic interactions can be developed between oppositely charged proteinic fragments which are then fold to form the fibrous network.30 This hypothesis also suggests that together with the proteins cross-linking, a glass transition process concurs due to the removal of water during the mechanical stretching, which is used as a plasticizer for the system.30

The hypothesis of non-covalent ionic interactions between the building blocks of the slime was further analysed by Baer et al.6 According to the authors, supramolecular non-covalent interactions between the lipid-protein nanoglobules are the driving force for the formation of fibers.6 Initially, the proteinic nanoglobules that compose the crude slime are colloidally stable due to mutual electrostatic repulsions. Subsequently, the formation of aggregates and hence stiff proteinic fibres is a result of colloidal instability that is caused by the mechanical shearing.47 After a series of experiments, this group found that the formation of nanoglobules and the ability to draw fibres are well correlated and that both depend highly on the pH and ionic strength of the slime solution.47 More specifically, the increase of ionic strength and the variation on pH were shown to lead to dissociation of nanoglobules and destruction of the ability for fibres formation.47 These observations could indicate screening effects on the
nanoglobules, that agrees with the observation that the slime does not harden in seawater\textsuperscript{35}, and therefore a proof for the electrostatic nature of the developed interactions.\textsuperscript{47}

Moreover, by the identification of phosphorylated proteins and divalent metal ions (calcium and magnesium) in the biochemical composition of the slime,\textsuperscript{47} it was suggested that specific ionic bindings between the high molecular weight proteins could drive to the formation of globular and fibrous structures.\textsuperscript{47} In Fig. 9, the proposed by Baer et al.\textsuperscript{47} electrostatic attractions which drive the self-assembly of nanoglobules are schematised. During mechanical shearing, the created threads bring the proteinic nanoglobules in such proximity that bridging between phosphate groups of adjacent proteins can be developed through calcium and electrostatic interactions between phosphate group and cationic group, and finally between cationic-anionic fragments can be generated.\textsuperscript{47}

What is not yet clear and is not discussed in the literature is the induced dehydration process of the slime after mechanical tension. We hypothesize that mechanical shear in the crude slime may induce a change in the dominancy of the interactions in the system. Initially, the system is stabilized due to hydrophilic interactions and mutual electrostatic repulsions that prevent proteins from folding, as the proteins of the velvet worm slime contain less amount of hydrophobic residues than typical globular proteins (Table I). After the application of stretching in the system, electrostatic attractions between proteinic residues overlap the hydrophilic repulsions and the system becomes more hydrophobic by forming supramolecular fibrous assemblies. At that moment, the system may push out water molecules forming a dry stiff material. However, more investigations should be performed in order to soak light in the complicated mechanism that drives the natural phase transition of velvet worm slime.

5. Conclusions and Perspectives

With this review, we aim to build an understanding around the properties that characterize the hunting weapon of velvet worms. We comprehensively presented all the related aspects which can affect and explain the properties of this biological material. The adhesive slime of velvet worms could be an inspiration for synthetic bio-compatible polymeric materials, not only in terms of material properties, but also in terms of material fabrication strategy. In this review, we have seen how nature itself produces such an intelligent
material well-adapted under the circumstances of an ambush predator that live in humid microclimates. The fact that the velvet worm bio-adhesive material is formed out of the body without bio-medication control inside the organism, as is the case of silks and mussel byssus, gives us inspiration for the transfer of that mechanism in the manufacture of new synthetic polymer systems.

Indeed, the extracellular mechano-chemical stimuli that drive the formation of that extraordinary biopolymeric material can be an inspiration for new sustainable stimuli-responsive control over structure strategies in the synthesis of advanced polymers. The fact that a material composed mainly from proteins and water folds into a glassy elastomer of the class of the synthetic manmade Nylon under ambient conditions makes us think the development of more environmentally friendly and bio-compatible materials. For instance, the controllable phase transition of the velvet worm slime can be an inspiration for future polymeric recyclable materials, that can degrade under environmentally friendly conditions into naturally perishable. This material could also be used as a universal adhesive because of its ability to adhere to plastic, metal, glass and biological surfaces.

Furthermore, such a natural stiff material could be an inspiration for new generation bio-compatible materials, that could replace the conventional plastic or be used for inside the body applications. During the degradation of such material, biological friendly blocks would be extracted intra-body. In addition, such a mechanoresponsive material could also be applicable as an organic actuator under conditions of high pressure in dry or humid microenvironments for intra or out of the body applications. For instance, a material inspired from the properties of velvet worms slime could be used as bleeding control material in skin traumas. Due to the high pressure of the ejected blood, such a coated material would lead to spontaneous solidification and stop bleeding until the recovery of the tissue.

At the end of this study, we conclude with the prevailing view between researchers that after mechanical drawing, electrostatic supramolecular interactions are developed between the intrinsically disordered proteins that compose the slime. As a result, fast self-assemblies, in the form of fibrous material, are formed, which can then dissociate in its building blocks after rehydration. However, the exact mechanism that drives and characterizes this supramolecular bioassembly is not yet fully clear. We are convinced that researchers are still in early stages in the level of understanding of this complex system, and we believe that a lot of different points need more investigation.

Firstly, we assume that the nature of the intra-specific interactions inside the nanoglobules building blocks is not yet clear enough, and analytical investigations should be performed. In this manner, a better understanding of the factors that lead to the formation of the monodispersed nanoparticles in the undisturbed slime would be gained. Moreover, the role of lipids, sugars and surfactants in combination with proteins in the synergetic mechano-chemical process of fibres formation is not yet clear and could be the key to build up an integrated model of the fibre’s formation.

In addition, we observe a lack of knowledge and discussion around the induced by the mechanical shearing dehydration process. Indeed, no discussion was also found around the dissolution of fibres in their building blocks after washing with distilled water. We assume that this process can indicate the type of interactions that are developed in the adhesive material during mechanical tension. For the understanding of this process, we believe that a careful use of the laws of thermodynamics and the theory about entropic elasticity and elastomeric response could give answers. However, this discussion is out of the scope of the present work and is proposed as a topic for future investigation.

Overall, we strongly believe that the bio-adhesive material of velvet worms presents unique properties that make it highly relevant for the fabrication of bio-inspired synthetic polymer systems. However, a lot of unknown sites should be studied further in order to reach a level of understanding able to move on new biomimetic strategies.

References
The Slimy Deadly Weapon of Velvet Worms: an Inspiration for Bio-Compatible Adhesive Materials

28. Lengerer, B. et al. Biological adhesion of the flatworm Macrostomum lignano relies on a


The Slimy Deadly Weapon of Velvet Worms: an Inspiration for Bio-Compatible Adhesive Materials


