Caractérisation des hémocytes de *Sepia* officinalis et développement de leur culture primaire dans le but de réaliser des tests de toxicité in vitro

### Préambule

Cette partie rapporte les études réalisées sur les hémocytes de la seiche commune.

Le chapitre six présente la caractérisation des hémocytes de *S. officinalis*, pré-requis indispensable à toute étude approfondie de ce type cellulaire. Dans ces travaux, nous avons cherché à 1) confirmer les observations d'études précédentes par des outils jamais utilisés sur ce type cellulaire (i.e. cytométrie en flux, microscopie électronique), et 2) affiner la compréhension du fonctionnement du système immunitaire de ce céphalopode par différents dosages enzymatiques (i.e. phosphatases acides, lysozymes, POs, inhibiteurs de protéases) dans les compartiments de l'hémolymphe (i.e. plasma, hémocytes), associés à l'étude de la phagocytose.

Le chapitre sept décrit les tests réalisés afin de 1) déterminer des paramètres de culture *in vitro* (i.e. milieu, température, et temps de culture), et 2) étudier les éventuelles perturbations de la phagocytose et de la production de ROS sur ces cellules, induites par des expositions à différents éléments traces (i.e. Cd et Zn).

## Chapitre 6

# Hemocyte morphology and phagocytic activity in the common cuttlefish (*Sepia* officinalis)

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#### Abstract

Little is known about the immune system of cephalopods, in spite of their many highly derived characters within the molluscan clade, including a vertebrate-like high-pressure closed circulatory system. Further the economic importance of cephalopod fisheries, potential for aquaculture, and use as ecotoxicology models demand a thorough understanding of their immune system. In this study, we present a comprehensive characterization of hemocytes in the common cuttlefish *S. officinalis*. Cytological stainings, electron microscopy- and flow cytometry-observations highlight a single granulocyte population with various densities of of eosinophilic granules and unstained vesicles. These hemocytes contain acid phosphatase-, lysozyme- and proPO system enzymes, and have high activity in bead phagocytosis assays. Interestingly, bead pre-incubation in plasma results in time-dependent aggregation perhaps resulting from hemocyanin-coating, and decrease in phagocytosis. This study provides the basis for understanding hemocyte-mediated immunity in the common cuttlefish, and essential background for future studies on cephalopod immunity.

**Keywords :** Cuttlefish (*Sepia officinalis*); Cephalopod mollusk; Hemocyte population; Enzymatic assay; Phagocytosis; Plasma opsonization

## 6.1 Introduction

Invertebrates resist pathogens despite their lack of adaptive immunity (Loker *et al.*, 2004). The tremendous variety of invertebrate life histories and ecological niches suggests a great diversity of immune strategies (Rowley et Powell, 2007). Among Mollusca – one of the most diverse groups of animals (Ponder et Lindberg, 2008), studies of immunity have mostly focused on bivalves (e.g. Aladaileh *et al.*, 2007; Allam *et al.*, 2002; Chang *et al.*, 2005; Donaghy et Volety, 2011; Donaghy *et al.*, 2009a; Hong *et al.*, 2013; López *et al.*, 1997b; Matozzo *et al.*, 2007; Salimi *et al.*, 2009; Wang *et al.*, 2012; Wootton et Pipe, 2003) and gastropods (e.g. Accorsi *et al.*, 2013; Barracco *et al.*, 1993; Donaghy *et al.*, 2010; Mahilini et Rajendran, 2008; Matricon-Gondran et Letocart, 1999; Travers *et al.*, 2008), while few studies have focused on cephalopods (Castellanos-Martínez et Gestal, 2013; Ford, 1992). Cephalopods are an interesting model because of their (1) vertebrate-like high-pressure closed circulatory system, (2) high sensitivity to environmental parameters, (3) short-life span and (4) elevated metabolic rate (Melzner *et al.*, 2007a; Pierce *et al.*, 2008). Moreover, their economical importance has recently grown in terms of fisheries and aquaculture (e.g. Berger, 2010; Payne *et al.*, 2006; Reid *et al.*, 2005; Sykes *et al.*, 2006b; Uriarte *et al.*, 2011).

As in other invertebrates, the cephalopod immune system relies on humoral factors and cell-mediated mechanisms acting together to eliminate invading micro-organisms (Soudant et al., 2013; Malham, 1996). Humoral factors mainly include lectins (e.g. agglutinins, opsonins), antimicrobial factors (e.g. peptides, acid phosphatases, lysozymes), and several signaling pathways including prophenoloxidase (proPO) and proteolytic cascades (Rowley et Powell, 2007; Wang et al., 2013b). In contrast, cell-mediated defense mechanisms are primarly performed by hemocytes (Hcs) – cells synthetized in white bodies and freely circulating in plasma and infiltrating in tissues (Claes, 1996; Cowden et Curtis, 1981). Hcs are of central importance to invertebrates because of their involvement in numerous physiological functions (Beninger et al., 2003; Donaghy et al., 2009b; Matozzo et al., 2001; Montes et al., 1995; Mount et al., 2004), including their ability to phagocytose, encapsulate and destroy foreign particles (Donaghy et al., 2010; Rodríguez-Domínguez et al., 2006; Soudant et al., 2013). In cephalopods, Hcs have mainly been described as a one cell-type population with large lobate nucleus and abundant granules, able to phagocytose (Bolognari, 1951; Collins et al., 2012; Cowden et Curtis, 1981; Malham, 1996; Rodríguez-Domínguez et al., 2006). However, these

Hc descriptions were mainly performed in Octopodidae as well as in the sepiolidae *Euprymna* scolopes and little is known about the immune cellular factors of other cephalopod species such as the sepiidae (cuttlefish) Sepia officinalis. Because of the distinct ecology of Sepiidae within Cephalopodia (Boucaud-Camou et Boismery, 1991; Uriarte *et al.*, 2011; Wang *et al.*, 2003), they may also have distinct immune requirements.

In this study, we performed cytological stainings, electron microscopy and flow cytometry (FCM) analysis to morphologically characterize *S. officinalis* Hcs. In addition, we investigated humoral factor localization between plasma and cells, and phagocytic reactions at several incubation times, temperatures and plasma concentrations. Our results highlighted one granulocyte population with various densities of acidophilic granules and unstained vesicles. The Hcs, which contained acid phosphatase, lysozyme and proPO system enzymes, had high phagocytic ability, modulated by plasma in our assay conditions.

## 6.2 Material and methods

#### 6.2.1 Animals

Thirty-one adult cuttlefish S. officinalis  $(21.5 \pm 3.1 \text{ cm} \text{ mantle length})$  were obtained from traps deployed during spring 2011 and 2012 along the Calvados coast (Normandy, France). Cuttlefish were then conditioned at the Centre de Recherches en Environnement Côtier (Lucsur-Mer, Normandy, France) in 4500-L tanks in an open seawater circuit for at least 2 days, fed with crustaceans *Crangon crangon* and *Carcinus maenas*, and starved for 1 day before experimentation. The sex of each individual was determined.

#### 6.2.2 Hemolymph collection

Before hemolymph sampling and following ethical procedures (Directive 2010/63/EU), cuttlefish were anesthetized as described by Andrews *et al.* (2013) through placement for 10 min in seawater containing 2 % ethanol. Between 9 and 13 ml of hemolymph was then withdrawn from the anterior mantle vein (King *et al.*, 2005) using a syringe with 18-gauge needle. The sample was transferred into a sterile tube, diluted or not with one volume of cooled sterile antiaggregative modified Alsever solution (115 mM glucose; 27 mM sodium

citrate; 11.5 mM EDTA; 382 mM NaCl pH 7.5; Bachère *et al.*, 1988), depending on the procedure (see below), and kept on ice to minimize cell clumping. Hc viability was checked by mixing one volume of Alsever-diluted hemolymph with one volume of trypan blue solution (0.4 %) and Hc concentration was determined with non diluted hemolymph using a Thoma cell. Once sampling was completed, animals were euthanized by increasing ethanol concentration to 10 % (Oestmann *et al.*, 1997).

#### 6.2.3 Chemicals

Sodium chloride (NaCl), anhydrous and hexahydrate magnesium chloride (MgCl<sub>2</sub> and MgCl<sub>2</sub>,6H<sub>2</sub>O), calcium chloride (CaCl<sub>2</sub>), bovin serum albumin (BSA), Bradford reagent, trypsin TPCK (N-Tosyl-L-phenylalanine chloromethyl ketone), N $\alpha$ -benzoyl-L-arginine 4-nitroanilide hydrochloride (BAPNA), p-nitrophenyl-phosphate, dimethyl sulfoxide (DMSO), trizma hydrochloride (Tris-HCl), trizma base, 3,4-dihydroxy-L-phenylalanine (L-DOPA), tropolone, hen egg white (HEW) lysozyme, freeze-dried *Micrococcus lysodeikticus*, sodium phosphate dibasic dihydrate (Na<sub>2</sub>HPO<sub>4</sub>,  $2H_2O$ ), citric acid (C<sub>6</sub>H<sub>8</sub>O<sub>7</sub>), Wright stain, neutral red, L-15 medium (Leibovitz), potassium chloride (KCl), magnesium sulphate heptahydrate (MgSO<sub>4</sub>,7H<sub>2</sub>O), formaldehyde solution, L-glutamine, streptomycin, sodium hydroxide (NaOH), ethylenediaminetetraacetic acid (EDTA), trypan blue solution, methanol and HEPES were obtained from Sigma-Aldrich (France). Halt Protease Inhibitor Cocktail, EDTA-Free (100X) was obtained from Thermo Fisher Scientific (Waltham, USA). Ethanol was obtained from Carlo erba (Milan, Italy). Hemacolor<sup>®</sup> staining kit was obtained from Merck Millipore (Darmstadt, Germany). Low melting point agar was obtained from Carl Roth (Lauterbourg, France). All chemicals used for electron microscopy i.e. glucose, sodium citrate, glutaraldehyde, sodium cacodylate, sucrose, osmium tetroxide  $(OsO_4)$ , propylene oxide, araldite resin, uranyl acetate and lead citrate were obtained from Electron Microscopy Sciences (Hatfield, PA, USA).

#### 6.2.4 Morphological characterization of *S. officinalis* Hcs

#### 6.2.4.1 Hemolymph cell monolayer stainings

For Hc staining, one drop of hemolymph was placed on a Thermanox<sup>TM</sup> coverslip (Thermo Fisher Scientific, Waltham, USA) and allowed to adhere for 30 min at 15°C. Before staining, coverslips were rinsed in Molluscan Physiological Saline (MPS; 0.4 M NaCl, 0.1 M MgSO<sub>4</sub>, 20 mM HEPES, 10 mM CaCl<sub>2</sub> and 10 mM KCl modified after Lowe *et al.* (1995)) to remove plasma. Hemacolor<sup>®</sup> staining was performed according to the manufacturer's recommendations. Wright staining was performed after 1 min air-drying by 1 min dehydration in absolute methanol, following by 1 min in Wright solution (0.66 % in methanol), then diluted (1:4) in distilled water during 4 min and rinsed. To highlight the cell lysosomal system, neutral red (NR) uptake was performed by incubating cells for 30 min in NR solution (1:500; NR stock solution (20 mg NR/ml DMSO):MPS) in a moist chamber at 15°C, before observation.

Freshly spread Hcs were observed using an inverted binocular microscope (Leica<sup>®</sup> DM IRB, Leica Microsystems, Wetzlar, Germany). Stained Hc observations were carried out with a Nikon Eclipse 80i light microscope with computer-assisted microscopic image analysis system, NIS-elements D 2.30 software (Nikon<sup>®</sup>, Champigny-sur-Marne, France).

#### 6.2.4.2 Electron microscopy

After 10 min 300 × g centrifugation, Hc pellets (N = 5) were rinsed with MPS and fixed in 3.2 % glutaraldehyde in 0.31 M sodium cacodylate buffer, with 0.25 M sucrose (pH 7.4) during for 90 min at 4°C. The cells were washed 3 times in rinsing solution (0.4 M sodium cacodylate, 0.3 M sucrose, pH 7.4). Then, cells were post-fixed 1 h with 1 % OsO<sub>4</sub> in cacodylate buffer 0.2 M, with 0.36 M sucrose (pH 7.4) at 4°C (protected from light), and washed in rinsing solution.

For scanning electron microscopy (SEM), cells were sedimented for 7 days on Thermanox<sup>TM</sup> coverslip coated with poly-l-lysine (Thermo Fisher Scientific, Waltham, USA) during several days. They were then dehydrated in progressive bath of ethanol (70-100 %) and critical point dried (Leica<sup>®</sup> EM CPD030). Finally, cells were sputtered with platinum and observed with scanning electron microscope JEOL 6400F. Freshly sampled cell diameters were determined by measuring 100 cells per cuttlefish (N = 5).

For transmission electron microscopy (TEM), cells were pellet in 2 % low melting point agar at 40°C, and then dehydrated through increasing concentrations of ethanol (70-100 %) and propylene oxide 100 %, embedded in araldite resine and allowed to polymerise for 48 h at 60°C. Ultrathin sections were done and contrasted with 2.5 % uranyl acetate diluted in 50 % ethanol for 30 min and contrasted for 5 min in Reynold's lead citrate (Venable et Coggeshall, 1965). Finally, cells were observed by transmission electron microscope JEOL 1011 and images were obtained with Camera Megaview 3 and Analysis Five software.

#### 6.2.5 Biochemical analysis

#### 6.2.5.1 Enzyme extraction

Hcs and plasma were separated in non diluted hemolymph by  $500 \times g$  centrifugation for 10 min at 4°C. Plasma was then removed, checked for absence of Hc microscopically, and stored in aliquots at -80°C until analysis. Upon complete plasma removal, Hc pellets were gently rinsed in either Tris buffer pH 7 (0.1 M Tris-HCl, 0.45 M NaCl, 26 mM MgCl<sub>2</sub> and 10 mM CaCl<sub>2</sub>) for phenoloxidase (PO) assays (Luna-Acosta, 2010) or Tris buffer pH 8 (10 mM Tris-HCl and 150 mM NaCl) for acid phosphatase, lysozyme and protease inhibitor (PI) assays (Safi, 2013). Cells were resuspended at  $10 \times 10^6$  cells mL<sup>-1</sup> in same extraction buffer and sonicated at 60 W for 20 s. Cell extracts were then centrifuged at  $10,000 \times g$  and their supernatant aliquoted and stored at  $-80^{\circ}$ C until analysis.

#### 6.2.5.2 Enzymatic assays

All activities were expressed in relation to protein concentration measured by the Bradford method (Bradford, 1976) using BSA as standard.

Total acid phosphatase activity was determined according to Moyano *et al.* (1996) using p-nitrophenyl-phosphate 2 % as substrate in a 1 M Tris buffer at pH 3. Then, 10  $\mu$ L of supernatant was added to 10  $\mu$ L of substrate in 96-well flat bottom plates (BD, USA). After 30 min incubation at 25°C, 100  $\mu$ L of NaOH 1 M were added to stop the reaction. The absorbance was measured at 405 nm using a Mithras LB 940 luminometer (Berthold, Thoiry, France). Total acid phosphatase activity was expressed as specific activity (U mg<sup>-1</sup> protein) where one enzymatic unit corresponded to the absorbance recorded after incubation.

Lysozyme activity was quantified according to Malham *et al.* (1998b). Fifty  $\mu$ L of HEW lysozyme (85  $\mu$ g mL<sup>-1</sup> in Tris buffer pH 8 described in section 2.5.1.) standard was serially diluted in triplicate in 96-well flat bottom plates (BD, USA). Fifty  $\mu$ L of each sample was added in triplicate to the 96-well plates as well as 50  $\mu$ L of Tris buffer pH 8, as blanks. One hundred and fifty  $\mu$ L of the substrate, freeze-dried *M. lysodeikticus* (75 mg/100 mL of phosphate/citrate buffer pH 5.8 (Na<sub>2</sub>HPO<sub>4</sub>,2H<sub>2</sub>O, 4.45 g/250 mL distilled H<sub>2</sub>O; C<sub>6</sub>H<sub>8</sub>O<sub>7</sub>, 2.1

g/100 mL distilled H<sub>2</sub>O; NaCl, 0.09 g/100 mL buffer), was added to each well. The reductions in turbidity in the wells were read on Mithras LB 940 luminometer (Berthold, Thoiry, France) at 25°C for 5 minutes at 10 second intervals at 450 nm using negative kinetics. Lysozyme concentrations were calculated from the standard curve ( $\mu$ g HEW lysozyme equivalent mL<sup>-1</sup>). Final lysozyme-like activity was thus expressed as  $\mu$ g HEW lysozyme eq. mg<sup>-1</sup> protein.

As described by Malham *et al.* (1998b) and Thompson *et al.* (1995), PI activity was measured by transferring 20  $\mu$ L of sample and 10  $\mu$ L of trypsin TPCK (100  $\mu$ g mL<sup>-1</sup> of 0.05 M Tris buffer pH 8) in 96-well flat bottom plates (BD, USA), and mixed at room temperature for 5 minutes. In parallel, intrinsic trypsin activity was measured by replacing 10  $\mu$ L of trypsin by Tris buffer pH 8 described above (section 2.5.1.). A positive control was used by replacing the sample with Tris buffer pH 8. Two hundred  $\mu$ L of BAPNA substrate solution (5.2 mg BAPNA mL<sup>-1</sup> DMSO) in 10 mL of 0.01 M trizma base buffer pH 7.4) was added to each well and incubated for 15 minutes at room temperature. Absorbance was read at 405 nm using Mithras LB 940 luminometer (Berthold, Thoiry, France), and PI activity was expressed as the percentage of trypsin sample inhibition (TI) compared to the positive control.

In order to partly discriminate PO synthesis and activation site, we took care to avoid unwanted activation of proPO during each step of the experiment. PO-like activity was measured spectrophotometrically by recording the formation of o-quinones, as described by Luna-Acosta (2010) with slight modifications to distinguish artificially activated PO (APO) (corresponding to PO-like activity resulting from zymogenic PO (proPO) activation plus already 'active' form) and in vivo 'active' PO form (Le Pabic, 2014). PO assays were conducted in 96-well flat bottom plates (BD, USA). L-DOPA was used as substrate, at a final concentration of 10 mM (Luna-Acosta, 2010) and prepared extemporaneously in Tris buffer pH 7 described above (section 2.5.1.). Tropolone (10 mM) and trypsin TPCK (1 g L<sup>-1</sup>) were used respectively as PO inhibitor and elicitor as previously described in S. officinalis (Lacoue-Labarthe et al., 2009a; Le Pabic, 2014). To avoid uncontrolled proPO activation by intrinsic proteases, Halt Protease Inhibitor Cocktail, EDTA-Free (1X) wide spectrum PI was used. For each sample, autoxidation of sample, 'basal', 'inhibited' and 'activated' PO-like activities were measured. For non-enzymatic sample autoxidation, 10  $\mu$ L of sample was mixed with Tris buffer pH 7. For 'basal' PO-like activity, 10  $\mu$ L of sample was firstly mixed during 10 min with 1.4  $\mu$ L Halt Protease Inhibitor Cocktail (100X), followed by adapted volume of Tris buffer pH 7 and 80  $\mu$ L L-DOPA. Similarly, for inhibited or APO-like activity, 10  $\mu$ L of sample was mixed with 10  $\mu$ L of tropolone (140 mM) or trypsin TPCK (14 g L<sup>-1</sup>), Tris buffer pH 7 and 80  $\mu$ L L-DOPA. Each measurement was systematically controlled by replacing sample by buffer, always in a final volume reaction of 140  $\mu$ g. Immediately after L-DOPA addition, PO-like activities were monitored at 25°C for 5 h using Mithras LB 940 luminometer (Berthold, Thoiry, France) at 490 nm (Luna-Acosta, 2010). When an inhibited PO-like activity was measured, this value was subtracted from APO and PO-like activities. Tropolone, with its copper chelator and peroxidase substrate properties, ensured that PO-like activity alone was detected (and not peroxidase). Results were also systematically corrected for non-enzymatic autoxidation of the substrate and were expressed in enzyme unit (1 U) per mg of total protein. One U corresponded to an increase of 0.001 in the absorbance per min at 25°C (Le Pabic, 2014).

#### 6.2.6 Flow cytometry (FCM) analysis

FCM analyses were performed using a Gallios flow cytometer (Beckman Coulter). In our study, excitation light was provided by a 22 mW blue diode (488 nm), and fluorescence was collected in the FL1 channel with a 525 nm bandpass filter. For each sample about 20,000 events were acquired. Data were analysed using Kaluza software (Beckman Coulter).

#### 6.2.6.1 Freshly sampled Hc cytomorphology

Hc morphology was based upon relative flow-cytometric parameters, Forward Scatter (FSC) and Side Scatter (SSC). FSC and SSC commonly measure particle size and internal complexity, respectively. Internal complexity, also reported as granularity, depends upon various inner components of the cells including shape of the nucleus, amount and types of cytoplasmic granules, cytoplasmic inclusions and membrane roughness (Donaghy *et al.*, 2012). Freshly withdrawn Hc was pelleted by  $300 \times g$  centrifugation for 5 min at 4°C, rinsed in MPS, before to be fixed in 3.7 % formaldehyde in MPS and kept in dark at 4°C until FCM analysis.

#### 6.2.6.2 Phagocytosis assays

Evaluation of phagocytosis was based on the ingestion of carboxylate-modified FluoSpheres<sup>®</sup> beads (1.0  $\mu$ m, yellow-green carboxylate-modified FluoSpheres<sup>®</sup>, Molecular Probes) by Hcs. Phagocytosis was expressed as (*i*) the percentage of cells that had ingested three or more microbeads (Auffret *et al.*, 2006; Hégaret *et al.*, 2003; Allam *et al.*, 2002), and (*ii*) the average number of microbeads per phagocytic Hcs (Donaghy *et al.*, 2010).

Hemolymph-diluted Alsever solution was used to plate Hcs at  $1.0 \times 10^6$  cells per well in 6-well plates, into which three volumes of sterile artificial seawater (25.5 g L<sup>-1</sup>, NaCl, 6.4 g L<sup>-1</sup>, MgSO<sub>4</sub>, 5.2 g L<sup>-1</sup> Hepes, 1.1 g L<sup>-1</sup> CaCl<sub>2</sub>, 0.75 g L<sup>-1</sup> KCl pH 7.4) were added to allow cell adhesion. After 60 min of incubation in dark at 15°C, supernatant was removed and cells were covered with 1 mL modified sterile Leibovitz L-15 medium pH 7.6 (20.2 g L<sup>-1</sup> NaCl, 0.54 g L<sup>-1</sup> KCl, 0.6 g L<sup>-1</sup> CaCl<sub>2</sub>, 1 g L<sup>-1</sup> MgSO<sub>4</sub>,7H<sub>2</sub>O and 3.9 g L<sup>-1</sup> MgCl<sub>2</sub>,6H<sub>2</sub>O) (Domart-Coulon *et al.*, 1994), supplemented with 2 mM L-glutamine, 100  $\mu$ g mL<sup>-1</sup> streptomycin and 60  $\mu$ g mL<sup>-1</sup> penicillin G into which FluoSpheres<sup>®</sup> were added at a ratio of 1:100 (Hc:beads). Hcs from same individual cuttlefish were analyzed at each time.

#### In vitro impact of time and temperature

Hcs were collected from 5 cuttlefish as described in section 2.2. To determine temperaturedependence of *S. officinalis* phagocytosis, three different temperatures were investigated; one physiological temperature (15°C) and two temperatures (4 and 25°C) out of the thermal window determined by Melzner *et al.* (2007b) with 15°C-acclimated cuttlefish. In this context, media with FluoSpheres<sup>®</sup> were acclimated to different working temperatures before addition to Hcs. Then, 6-well plates were placed in dark at 4, 15 and 25 CO<sub>2</sub>-free incubators, during 30, 60, 120 and 180 min incubation. Afterwards wells were rinsed with MPS to remove bead excess, following by gentle scrapping and centrifugation at  $300 \times g$  for 5 min at 4°C. Resulting supernatants were removed, pellets resuspended in 3.7 % formaldehyde solution in MPS and stored in the dark at 4°C until FCM analysis.

#### In vitro impact of plasma concentrations

To study the plasma opsonization process,  $FluoSpheres^{\mathbb{R}}$  were added to modified L-15 medium mixed with cell-free plasma at 0 (as control), 1, 10 and 50 %. These mixtures were

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incubated at 15°C during 1 h before homogenization and addition to cells. After 2 h incubation at 15°C, cells were treated as previously described for FCM analysis.

In parallel, bead phagocytosis was performed in modified L-15 medium or 100 % plasma, and Hcs analysed by TEM as described in section 6.2.4.2

#### 6.2.7 Data analysis

Residual distributions were tested for normality (Shapiro test) as well as homogeneity of variances (Levene test). Student's t-test was used to compare Hc concentrations between male and female individuals, specific activities between Hc and plasma compartments, PO- and APO-like activities in each compartment, and phagocytic parameters between control and several plasma treatments. One-way analysis of variance (ANOVA) followed by non-parametric pairwise permutational t-tests (N < 30) was used to determine the impact of temperature on phagocytic parameters at each time. Analysis of covariance (ANCOVA) was used to compare phagocytic parameter time-evolution as function of the temperature. All results are expressed and displayed as mean  $\pm$  standard deviation (SD). The statistical significance was designed as being at the level of p < 0.05. R software was used for statistics and graphics.

Species	N	Concentration	Range	Authors	
Cephalopod					
Loligiginidae					
$Sepioteuthis\ lessoniana$	18	$2.80 \pm 4.24$	-	Heming et al. (1990)	
Sepiidae					
$Sepia \ officinal is$	31	$7.23\pm5.62$	0.92 - 20.31	Present study	
Sepiolidae					
$Euprymna\ scolopes$	-	5.0	-	Collins et Nyholm $(2010)$	
Octopodidae					
Eledone cirrhosa	$\geq 5$	> 10.0	-	Malham <i>et al.</i> (1998a, 2002)	
Octopus vulgaris	28	$10.67 \pm 7.32$	2.3 - 25.0	Rodríguez-Domínguez <i>et al.</i> (2006)	
O. vulgaris	92	$10.3 \pm 7.77$	0.49 - 32.0	Castellanos-Martínez $et \ al. \ (2014)$	
Castropod					
Ampullariidae					
Pomacea canaliculata	3	$1.1 \pm 0.1$	_	$A_{ccorsi}$ et al. (2013)	
Heliotididee	5	$1.1 \pm 0.1$	-	Accorsi et $ui. (2013)$	
Haliotia discus discus	38	2.24	_	Donaghy et al. $(2010)$	
Haliotis tuberculata	40	$6.0 \pm 0.7$	_	Travers <i>et al.</i> $(2008)$	
Planorbidae	40	0.0 ± 0.1			
Biomphalaria tenagophila	10	$0.25 \pm 0.13$	0.11 - 0.55	Barracco $et al.$ (1993)	
Turbinidae	10	0.20 ± 0.10	0.11 0.00		
Turbo cornutus	35	1.50	-	Donaghy et al. $(2010)$	
Bivalve					
Cardiidae					
$Cerastoderma\ edule$	10	$4.54 \pm 2.21$	-	Wootton et al. $(2003)$	
Cerastoderma glaucum	10	$0.55\pm0.22$	-	Matozzo et al. (2007)	
Mytilidae					
Mytilus edulis	10	$5.68 \pm 3.63$	-	Wootton $et al.$ (2003)	
Perna perna	60	$3.41 \pm 1.77$	-	Barracco et al. (1999)	
Perna viridis	20	$1.30 \pm 0.35$	0.73 - 2.20	Donaghy et Volety (2011)	
P. viridis	6	$5.54 \pm 1.30$	-	Wang et al. (2012)	
Ostreidae					
$Crassostrea\ ariakensis$	15	$0.71\pm0.22$	0.33 - 1.23	Donaghy et al. (2009a)	
$Crassostrea\ virginica$	-	-	0.65 - 2.80	Goedken et De Guise $(2004)$	
Pectinidae					
$Argopecten\ irradians$	$\geq 20$	37.9	-	Zhang <i>et al.</i> (2006)	
Pharidae					
Ensis siliqua	10	$6.48 \pm 2.50$	-	Wootton $et al.$ (2003)	
Semelidae					
$Scrobicularia\ plana$	$\geq 10$	2.99	-	Wootton et Pipe $(2003)$	
Tridacnidae					
Tridacna crocea	6	-	0.3 - 2.6	Nakayama <i>et al.</i> (1997)	
Unionidae					
Cristaria plicata	30	$2.37\pm0.51$	-	Xie <i>et al.</i> (2011)	
Veneridae					
Chamelea gallina	120	-	1.2 - 2.4	Pampanin <i>et al.</i> (2002)	
Macrocallista nimbosa	41	$1.08 \pm 0.47$	0.12 - 2.06	Donaghy et al. (2012)	
M. nimbosa	40	$0.99 \pm 0.52$	0.26 - 2.2	Jauzein <i>et al.</i> (2013)	

TABLE 6.1 – Reported circulant hemocyte concentrations (mean  $\pm$  SD and range  $\times 10^6$  cell mL<sup>-1</sup>) in cephalopod, gastropod and bivalve mollusks.

In *italic* : SD calculated from SE or  $CI_{95}$ 

## 6.3 Results and Discussion

Little is known about cephalopod Hcs, while Hc populations have been extensively studied in other mollusks (bivalves and gastropods). Our study provides the first-description of the circulating Hc population in the cuttlefish *S. officinalis* using FCM and electron microscopy. We also report biochemical- and phagocytosis analysis of Hc interaction with plasma.

#### 6.3.1 Hc viability and concentration

Hc viability in all cuttlefish used in this study was higher than 99 % (data not shown), and no Hc concentration difference was observed between males  $(7.20 \pm 5.70 \times 10^6 \text{ cells} \text{mL}^{-1}; N=18)$  and females  $(7.27 \pm 5.75 \times 10^6 \text{ cells mL}^{-1}; N=13)$ . Consequently, we obtained a mean Hc concentration in hemolymph of *S. officinalis* of  $7.23 \pm 5.62 \times 10^6$  cells mL<sup>-1</sup> ranging from 0.92 to  $20.31 \times 10^6$  cells mL<sup>-1</sup>. This mean concentration and concentration variability are consistent with reports on cephalopod Hcs (Table 6.1). Although highly variable, the Hc concentration of *S. officinalis* appeared slightly higher than that of the loliginidae *Sepioteuthis lessoniana*, similar to that of the sepiolidae *Euprymna scolopes*, and lower than that of Octopodidae, where the highest Hc concentrations within cephalopods are found (10  $\times 10^6$  cells mL<sup>-1</sup>) (Castellanos-Martínez *et al.*, 2014; Collins et Nyholm, 2010; Collins *et al.*, 2012; Heming *et al.*, 1990; Malham *et al.*, 2002, 1998a; Rodríguez-Domínguez *et al.*, 2006). Generally, Hcs of gastropod and bivalve mollusks appear less concentrated with most values ranging between 1 and 5  $\times 10^6$  cells mL<sup>-1</sup> (Table 6.1), albeit with population-dependent variation (Donaghy et Volety, 2011; Santarém *et al.*, 1994; Wang *et al.*, 2012).

#### 6.3.2 Hc identification and characterization

#### 6.3.2.1 Light microscopy

After adhesion, fresh cuttlefish Hcs rapidly displayed many thin pseudopodia, and most cells contained refringent and non-refringent granules of various densities in their cytoplasm (Fig. 6.1). Among different stains used, Wright's differential staining allowed the best observation of spontaneously adhering Hcs. A single cell type with large, lobate nucleus, slightly basophilic cytoplasm and acidophilic granules was identified (Fig. 6.2A). Lucent vesicles were also observed in the cytoplasm of some cells. NR uptake revealed dense lysosomal system in



FIGURE 6.1 – Freshly adhesive *Sepia officinalis* hemocytes presenting refringent (arrowhead) and non-refringent (arrow) granules. Inset : hemocytes completely spread



FIGURE 6.2 – Stained *S. officinalis* freshly adhesive hemocytes; (A) Wright staining highlighting large nucleus (n), slightly basophilic cytoplasm, acidophilic granules (arrow) and lucent granules (arrowhead) in spread hemocytes; (B) Neutral red uptake staining of two hemocytes highlighting lysosomal system.

most cells (Fig. 6.2B). According to the classification of bivalve Hcs, 2 main classes are usually accepted : granulocytes with cytoplasmic granules and hyalinocytes with few or no granules (Cheng, 1981). Based on the high granule density found in most Hcs, we classify *S. officinalis* Hcs as granulocytes. These observations are consistent with the majority of studies performed in Octopodidae (Bolognari, 1951; Cowden et Curtis, 1981, 1973; Kondo *et al.*, 2003; Malham, 1996), Sepiidae (Claes, 1996) and Sepiolidae (Collins *et al.*, 2012; Heath-Heckman et McFall-Ngai, 2011), which reported one granular cell population circulating in the hemolymph and also called macrophage-like Hc (Cowden et Curtis, 1981; Nyholm et McFall-Ngai, 1998).

#### 6.3.2.2 Electron microscopy

SEM observations of circulating cuttlefish Hcs showed a single cell type of  $10.3 \pm 0.8$   $\mu$ m in diameter and able to form numerous pseudopodia (Fig. 6.3A-B), consistent with the above described light microscopy observations. This cell size is consistent with the 8-10  $\mu$ m cell diameter measured on non-circulating mature Hcs in the cuttlefish white bodies (leukopoietic tissues) (Claes, 1996) and appeared slightly lower than Hc size of octopods *O. vulgaris* and *E. cirrhosa* Hcs : 11.6  $\pm$  1.2 and 12-15  $\mu$ m, respectively (Castellanos-Martínez *et al.*, 2014; Stuart, 1968).

TEM observations of circulating Hcs highlighted a large lobate nucleus without nucleoli but highly condensed chromatin mainly localized along the inner surface of the nuclear membrane (Fig. 6.3C). The cytoplasm of most cells contained various sizes and densities of two distinct inclusion types (Fig. 6.4A) : electron-dense granules corresponding to lysosomes (Claes, 1996), and electron-lucent membrane-surrounded vesicles, containing various densities of molecules with similar size and circular shape as Hcy molecules (Fig. 6.4B-C; (Bergmann *et al.*, 2006; Boisset et Mouche, 2000; Dilly et Messenger, 1972; Martoja et Marcaillou, 1993; Muzii, 1981)). These molecules are referred to as Hcy-like hereafter. In contrast to *E. cirrhosa* and *O. vulgaris* Hcs, neither different electron-dense granules nor lipid droplets were observed (Castellanos-Martínez *et al.*, 2014; Cowden et Curtis, 1981; Malham, 1996), suggesting different granule contents between these cephalopods. Mitochondria, Golgi apparatus and rough endoplasmic reticulum that often surrounded the nucleus were also observed in these cells (Fig. 6.3C inset).



FIGURE 6.3 – Electron micrographs of *S. officinalis* circulating hemocytes. (A) Scanning electron micrograph of several hemocytes showing similar aspect; (B) Scanning electron micrograph of one hemocyte producing many pseudopodia; (C) Transmission electron micrograph of circulating hemocytes presenting caracteristic lobate-nucleus (N) with highly condensed chromatin along the inner surface of the nuclear membrane, well-developped rough endoplasmic reticulum (rER), electron-dense lysosomal vesicles (arrow) and electron-lucent vesicles (arrowhead). Bar : 1  $\mu$ m. Inset : high magnification of mitochondria (m) and Golgi apparatus (G). Bar : 0.1  $\mu$ m.



FIGURE 6.4 – Electron micrographs of *S. officinalis* circulating hemocytes. (D) Transmission electron micrograph of circulating hemocytes presenting numerous vesicles; electron-dense lysosomal granules (arrow) and electron-lucent vesicles (arrowhead). Bar : 0.5  $\mu$ m. (E-F) Electron-lucent vesicles showing inner hemocyanin-like molecules (small arrow). Bars : 100 nm.

#### 6.3.3 Enzymatic assays

Hydrolytic enzyme lysozymes and acid phosphatases were detected in Hc extract alone (Table 6.2), consistent with their known lysosomal distribution (Blasco *et al.*, 1993; Fiołka *et al.*, 2012). These data are in agreement with carbohydrates and acid phosphatases detected by stainings in *E. cirrhosa* Hcs (Malham *et al.*, 1998a) and suggest their synthesis in *S. officinalis* Hcs or earlier during their maturation (Cowden, 1972; Malham *et al.*, 1998a). PI activities were exclusively found in plasma (Table 2), corresponding at least in part to the already described  $\alpha_2$ -macroglobulin activity – the second most abundant protein in *S. officinalis* plasma (Armstrong, 1992; Thøgersen *et al.*, 1992; Vanhoorelbeke *et al.*, 1994). Interestingly, PIs were recently reported in *E. cirrhosa* and *E. scolopes* Hcs (Collins *et al.*, 2012; Malham, 1996), underlying the need for future investigations.

Specific activity	Ν	Hemolymph compartment Hc	Plasma
Acid phosphatases (U mg prot <sup>-1</sup> ) Lysozymes ( $\mu$ g HEW lysozyme eq. mg prot <sup>-1</sup> ) PIs (tryspin inhibition %age) PO-like (U mg prot <sup>-1</sup> ) APO-like (U mg prot <sup>-1</sup> )	9 10 9 10	$\begin{array}{c} 23.5 \pm 10.6 \\ 21.9 \pm 9.0 \\ 0.0 \\ 20.3 \pm 9.5 \\ 30.5 \pm 7.4^* \end{array}$	$\begin{array}{c} 0.1 \pm 0.1 \\ 0.1 \pm 0.1 \\ 31.8 \pm 14.6 \\ 2.6 \pm 1.0 \\ 2.9 \pm 0.9 \end{array}$

TABLE 6.2 – Specific activity repartition in hemolymph compartments : hemocyte (10  $\times$  10<sup>6</sup> cell mL<sup>-1</sup>) and plasma. Asterisk (\*) indicates significance between PO- and APO-like activities in each compartment (p < 0.05).

PO-like activities were measured in both hemolymph compartments (Table 6.2). Siddiqui *et al.* (2006) showed in *S. officinalis* that plasma-associated PO-activity resulted from Hcy, which molecule shares a structurally and functionally equivalent active site with POs (Campello *et al.*, 2008). Therefore, PO-like activities found in Hc extracts probably result, at least in part, from Hcy presence in electron-lucent vesicles (TEM analysis). We note that PO activity was not previously detected in cephalopod Hcs. In contrast, proPO activation – the difference between PO- and APO-like activity, occurred in Hc extract alone (p=0.01).

#### 6.3.4 FCM analysis

#### 6.3.4.1 Fresh Hc cytogram

According to the criteria of cell size (FSC) and cell complexity (SSC), FCM supported the presence of a single Hc population in cuttlefish (Fig. 6.5A), albeit with variable internal complexity in some individuals (Fig. 6.5B), consistent with the above described TEM observations. Notably, recent *O. vulgaris* Hc characterization using TEM and CMF reported 2 circulating granulocyte morphs based on size, which were interpreted as different maturating stages of the same cell type (Castellanos-Martínez *et al.*, 2014). Consistently, a study of *S. officinalis* Hc synthesis suggested a Hc maturation model with a single cell precursor (Claes, 1996). This cephalopod organization is distinct from that of bivalves and gastropods, where most Hc studies reported several circulating Hc types derived from one or more cell precursors (e.g. Accorsi *et al.*, 2013; Donaghy *et al.*, 2010, 2009a; Hong *et al.*, 2013; Mahilini et Rajendran, 2008; Matozzo *et al.*, 2007; Prado-Alvarez *et al.*, 2012; Rebelo *et al.*, 2013; Salimi *et al.*, 2009; Travers *et al.*, 2008; Wang *et al.*, 2012). Our results further support the presence



of a single mature circulating cell type in the Hc population of cephalopods.

FIGURE 6.5 - Flow cytometer bivariate plots showing distributions of particle size (FSC) vs internal complexity (SSC) of fresh Hcs of S. officinalis. Insets representing histogram of both variables. (A) Typical cuttlefish dot-plot; (B) Dot-plot showing wide internal complexity (SSC) distribution sometimes observed.

#### 6.3.4.2 Phagocytosis experiments

#### 6.3.4.3 Time and temperature

Both phagocytosis parameters (i.e. percentage of phagocytic Hcs and quantity of ingested beads) appeared time-dependent at all temperatures tested (Fig. 6.6).

After 30 min,  $35.5 \pm 8.1$ ,  $40.1 \pm 6.6$ , and  $41.7 \pm 9.7$  % of cells were phagocytic at 4, 15 and  $25^{\circ}$ C, respectively, subsequently reaching  $63.3 \pm 7.2$ ,  $70.6 \pm 10.9$  and  $69.8 \pm 12.0$  % after 180 min (Fig. 6.6A). These phagocytic percentages are consistent with previous observations of Hcs in *O. vulgaris* and *E. cirrhosa* where 50 and 70 % of phagocytic cells were reported, respectively (Malham *et al.*, 1997; Rodríguez-Domínguez *et al.*, 2006). In contrast, a lower phagocytic percentage ( $\leq 13$  %) was recently reported for *O. vulgaris* Hcs after 120 min incubation at 15°C (Castellanos-Martínez *et al.*, 2014). The filtered seawater used as media during this *in vitro* experiment may account for this lower rate. Overall, our results are among the highest phagocytic percentages reported among mollusks, regardless of experimental conditions (e.g. Carballal *et al.*, 1997b; Cima *et al.*, 2000; Di *et al.*, 2013; Donaghy et Volety, 2011; Donaghy *et al.*, 2010, 2009a; Gorbushin et Iakovleva, 2007; Hong



FIGURE 6.6 – Graphs representing evolution of phagocytic parameters function of time (30, 60, 120 and 180 min) at three temperatures (4, 15 and 25°C) (N = 5); (A) Phagocytic hemocyte percentages and (B) engulfed bead number.

et al., 2013; Jauzein et al., 2013; Nakayama et al., 1997; Ordas et al., 1999; Prado-Alvarez et al., 2012; Travers et al., 2008; Wang et al., 2012; Wootton et Pipe, 2003; Xue et al., 2001).

The time-dependent evolution of phagocytosis measured in our study is consistent with previous findings on Hcs of mollusk bivalves (Canesi *et al.*, 2002; Cima *et al.*, 2000; Donaghy et Volety, 2011; Hong *et al.*, 2013; Ordas *et al.*, 1999) and gastropods (Adema *et al.*, 1991; Donaghy *et al.*, 2010; Travers *et al.*, 2008), but also crustacean (Raman *et al.*, 2008) and fish blood cells (Harford *et al.*, 2006). Within cephalopods, Malham *et al.* (1997) showed similar evolution on *E. cirrhosa* Hcs, whereas Rodríguez-Domínguez *et al.* (2006) reported constant *O. vulgaris* Hc phagocytic percentages from 45 to 120 min incubation at 22°C.

Notably, temperature sensitivity appears species-specific in cephalopods, as no effects were observed in our study, whereas negative effects of low temperature (4-10°C) were reported on Hc phagocytosis rate in *O. vulgaris* (Rodríguez-Domínguez *et al.*, 2006), and *E. cirrhosa* (Malham *et al.*, 1997). Although temperatures used were extremes and out of the thermal window of 15°C-acclimated cuttlefish (Melzner *et al.*, 2007b) (25°C - inducing anaerobic metabolism, and 4°C - a lethal temperature (Richard, 1971)), no significant effects on phagocytosis parameters were found in our study. This low effect of temperature on Hc phagocytosis ability is consistent with the eurythermy of *S. officinalis*. The quantity of ingested

beads measured by FCM evolved in similar ways as the percentage of phagocytic Hcs. Mean quantity of beads in Hcs increased during the entire experiment from  $6.0 \pm 1.1$ ,  $7.0 \pm 1.0$ and  $6.8 \pm 0.8$  beads/cell after 30 min to  $16.4 \pm 2.4$ ,  $18.7 \pm 4.9$  and  $16.9 \pm 4.1$  beads/cell after 180 min at 4, 15 and 25°C, respectively (Fig. 6.6B). Concurrent TEM observations confirmed the number of ingested beads registered by FCM (Fig. 6.7A) and visually demonstrated the engulfment process with (1) Hc-bead adhesion, (2) bead internalization by almost continuous close contact between membrane and particle, and (3) closing phagosome (Fig. 6.7B-C). Occasionally, lysosomal fusion with phagosome was also observed (Fig. 6.7D).



FIGURE 6.7 – Transmission electron micrographs of *S. officinalis* hemocyte. (A) Phagocytic hemocyte presenting several engulfed 1  $\mu$ m latex beads (b). Bar : 1  $\mu$ m. Sequential events of the internalization of beads (B-D); (B) hemocyte-bead adhesion. Bar : 0.1  $\mu$ m; (C) bead engulfment. Bar : 0.2  $\mu$ m; and (D) fusion between phagosomal and lysosomal compartments. Bar : 0.5  $\mu$ m.

Our phagocytosis assay revealed similar rates of bead engulfment as thoses found in *O.* vulgaris (Rodríguez-Domínguez *et al.*, 2006), but higher rates than in the mollusk bivalve Perna viridis and gastropods Lymnaea stagnalis, Haliotis discus discus and Turbo cornutus (Adema et al., 1991; Donaghy et Volety, 2011; Donaghy et al., 2010), highlighting higher engulfment capacity of cephalopod Hcs. Nevertheless, such differences could be explained by the high number of beads per cell used in our study (1:100) compared to those of previous ones ( $\leq 1:25$ ).

#### 6.3.4.4 Opsonization assay

As observed under FCM, opsonization experiments differently affected Hc phagocytosis percentage and engulfed bead number. While phagocytosis occurred without incubation of Hcs or beads in plasma, an increase in ingested bead number occurred after incubation in 1 or 10 % plasma (p=0.008 and 0.045, respectively) (Fig. 6.8). In contrast, incubation of beads and Hcs in 50 % plasma led to a decrease of phagocytosis percentage (p=0.04).



FIGURE 6.8 – Graph representing phagocytic hemocyte percentage and mean engulfed bead number of *S. officinalis* hemocytes ( $N \ge 4$ ; 2h incubation at 15°C) function of plasma concentration (%) treatments. Statistically significant differences were made against control (0% plasma concentration) at each plasma concentration for phagocytic cell percentage (°) and engulfed bead number (\*); \* and ° p<0.05; \*\* p<0.01.

TEM observations of phagocytosis conducted with plasma revealed a bead-coating by Hcy-like molecules (Fig. 6.9A-B) as previously suggested in cephalopods (Beuerlein *et al.*,

2002b; Malham et al., 1997; Stuart, 1968). This observation could explain Hcy presence in Hc electron-lucent vesicles. Moreover, Hcy-like molecule presence in bridges between beads (Fig. 6.9C) suggested an agglutinating function, consistent with the increasing engulfed bead number observed at 1 and 10 % plasma (Fig. 6.8). Such findings are consistent with a recent Octopus maya plasma agglutinin (OmA) characterization, showing homology between OmA subunits and one functional unit of Hcv from Octopus dofleini (Alpuche et al., 2010). Moreover, the bead engulfment increased after as low as 1 % plasma addition, underlying the high concentration of the molecule in charge of this process, and Hcy is known to represent more than 90 % of cephalopod plasma proteins (D'Aniello et al., 1986; Ghiretti, 1966; Malham et al., 1997; Mangold et Bidder, 1989a). Such agglutinating function could also explain the phagocytic percentage decrease occurring with 50 % plasma. Indeed, important bead agglutination might limit the availability of these particles for phagocytic process by increasing the size of particles to engulf. Ballarin et al. (1994) described a similar phagocytic decrease resulting from agglutinins in the ascidian *Botryllus schlosseri* after yeast pre-incubation in high plasma concentrations ( $\geq 50$  %). In cephalopods, this Hcy property could explain the decrease of phagocytosis by O. vulgaris Hcs measured after pre-incubation of zymosan in 100 % plasma (Rodríguez-Domínguez et al., 2006), because of zymosan particle (about 3  $\mu$ m) aggregation. Malham et al. (1997) and Ballarin et al. (1994) highlighted also the importance of phagocytose duration in this type of study, as phagocytosis enhancement after plasma incubation mainly occurred after 30 min. This is consistent with our study and that of Rodríguez-Domínguez et al. (2006) which report plasma-dependent phagocytosis decrease after 120 and 90 min of phagocytosis duration, respectively. Our results demonstrate the presence of a Hcy-like coating molecule with agglutinin function in S. officinalis plasma, which can modulate phagocytosis according to (1) pre-incubation plasma concentration and (2) phagocytosis duration.



FIGURE 6.9 – Transmission electron micrographs of 1  $\mu$ m latex beads (b). (A) One bead after incubation in medium without plasma add; (B) One bead after incubation in plasma presenting important coating by hemocyanin-like molecules (arrow). Bars : 100 nm. (C) Apparent hemocyanin-like molecule implication in bead aggregation (arrow). Bar : 200 nm.

## 6.4 Conclusions

Our study demonstrates, using FCM, light- and electron-microscopy, that a single granulocyte population with variable internal complexity circulates in the hemolymph of *S. officinalis*, as in other cephalopods. Acid phosphatase, lysozyme, and for the first time in cephalopods proPO system enzymes were detected in Hcs, but not in plasma (Malham, 1996; Grimaldi *et al.*, 2013). These cells were able to recognize and incorporate foreign material at high rate independently of temperature and without need for plasma. Concurrent TEM and FCM analysis suggested a role for Hcy in foreign particle coating probably associated with its hypothesized agglutinin function. These data provide important information to understand the Hc-mediated immunity in the common cuttlefish, and a useful background for future studies of cephalopod Hcs.

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