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The Cellular Basis of Platelet Secretion: Emerging Structure/Function Relationships

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Abstract

Platelet activation has long been known to be accompanied by secretion from at least three types of compartments. These include dense granules, the major source of small molecules; α -granules, the major protein storage organelle, and lysosomes, the site of acid hydrolase storage. Despite ~60 years of research, there are still many unanswered questions about the cell biology of platelet secretion: e.g., how are these secretory organelles organized to support cargo release and what are the key routes of cargo release, granule to plasma membrane or granule to canalicular system (CS). Moreover, in recent years, increasing evidence points to the platelet being organized for secretion of the contents from other organelles, namely, the dense tubular system (endoplasmic reticulum) and the Golgi apparatus. Conceivably, protein secretion is a wide-spread property of the platelet and its organelles. In this review, we concentrate on the cell biology of the α -granule and its structure/function relationships. We both review the literature and discuss the wide array of 3-dimensional, high resolution structural approaches that have emerged in the last few years. These have begun to reveal new and unanticipated outcomes and some of these are discussed. We are hopeful that the next several years will bring rapid advances to this field that will resolve past controversies and be clinically relevant.

Keywords

platelets; alpha-granules; secretion; platelet release reaction; microscopy

Introduction

Platelets are small, anucleate cell fragments that are central to thrombosis, hemostasis, and inflammation. They are derived from megakaryocytes from which they inherit their organelles. In the circulation, platelets can synthesize proteins and, as might be expected, they contain many of the cytoplasmic organelles found in most eukaryotic cells including endoplasmic reticulum [termed dense tubular system (DTS) in platelets], mitochondria, autophagosomes, endosomes, lysosomes, and in addition to these two platelet-specific organelles: the α -granule, the major site of protein storage in platelets, and the dense granule

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Declaration of Interest

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(aka δ -granules), the site of small molecule storage. In contrast to most cell types, almost all of the organelles listed above appear to be involved in secretion including DTS, lysosomes, and Golgi apparatus elements. Physiologically, the platelet is poised for exocytosis and can be triggered to rapidly release the contents of its organelles in response to such signals as collagen, thrombin or thromboxane A₂. In this review, we concentrate on the cell biology of the α -granule, the most abundant secretory organelle in platelets. We begin with a history of α -granule discovery, then consider the current status of this organelle and the role of emerging technologies in forcing a rediscussion of α -granule structure/relationships. In later portions of the article we consider protein secretion by non-conventional, platelet organelles such as the DTS and Golgi apparatus elements. Finally, we end with a perspective on routes to further understanding and the settlement of current α -granule structure/function relationship controversies. For a detailed consideration of dense granules and small molecule secretion by platelets, we refer the reader to a review by Ambrosio and di Pietro in this issue of the journal.

α -Granules: the Early Years

Three major experimental threads: structural biology (electron microscopy), protein localization (microscopy and cell fractionation), and platelet release studies, when drawn together resulted in the platelet α -granule being recognized as the major site of protein storage and subsequent stimulus dependent release. In brief, the initial discovery of platelet organelles and, in particular, α -granules dates back some decades to the beginnings of thin section electron microscopy in the 1950s. As such, the discovery was based on the size, shape and staining pattern of subcellular structures and, in current terms, could well be taken as an exercise in pattern recognition (see Figure 1A for example electron micrograph). As with all advances, the initial discovery is embedded in what now would be considered to be some degree of confusion. The title of an early publication illustrates this point: “*Electron microscopic studies of sectioned white blood cells and platelets, with observations on the derivation of specific granules from mitochondria*” [1]. Today we recognize that α -granules and other secretory organelles in cells are distinct in biochemistry and pathway of biogenesis from the mitochondria. However, at the time, this was not clear. We note that at first platelet granules were often referred to as granulomeres [2]. Moreover, as might be assumed from the present day use of the term α -granule and the less common use of the term δ -granule for dense granules, platelet organelles were originally named in a somewhat arbitrary order using the first few letters of the Greek alphabet. For example, α -granules were assigned the letter “ α ” because of their abundance and large size and the term “ β -granulomere” was assigned to mitochondria because they were prominent but much less abundant [3]. In addition to electron microscopy approaches, credit must be given to cell fractionation approaches to the establishment, by the early-to-mid 1960s, of the α -granules as the major organelle population within platelets [4].

Unfortunately, neither structural studies by thin section electron microscopy nor cell fractionation studies provide, by themselves, much clarity about the composition or function of platelet α -granules. Thin section electron microscopy records difference in electron density due to the binding of electron dense stains such as osmium tetroxide and uranyl acetate while cell fractionation scores the distribution of known proteins across a set of cell

fractions. Moreover, until the recent advent of mass spectrometric analysis, cell fractionation was limited to a very small number of candidate proteins assayed for enzymatic activity or immunoreactivity. Furthermore, the application of immunogold labeling [5] and fluorescence microscopy [6] starting in the 1970/80s as protein assignment tools were similarly restricted to a candidate protein approach. Antibodies, be they absorbed to gold particles for electron microscopy or fluorescently labeled and/or fluorescently labeled endocytic tracers such as tagged fibrinogen, were shown to accumulate in/over α -granules. Hence, by the late 1970s to the mid-1980s, a series of compositional studies showed that α -granules, in contrast to dense granules, were an organelle enriched in a small number of major adhesive proteins such as fibrinogen and von Willebrand factor (VWF) that are functionally important to platelet plug formation and the membrane protein, p-selectin.

The formal proof that platelet α -granules are the major site of agonist-dependent protein release by platelets and hence functionally important came from parallel platelet release reaction studies done over more than 3 decades. These studies which dates back to the middle 1950s initially used washed platelets (Figure 1B) and scored for serotonin release in response to thrombin [7–8] a dense granule cargo. Based on the outcome of such studies over two decades, the platelet release reaction was classified into two different processes, release reaction I (release of serotonin and ADP) and release reaction II (release of acid hydrolases, lysosomal proteins) [9–10]. This classification drew upon differences in agonist response, inhibitors, kinetics, metabolic requirements, and subcellular localization of the released molecules. As late as 1974, the localization of a major α -granule content protein such as platelet factor 4 (PF4) was sufficiently ambiguous that PF4 release, although clearly different from serotonin release, could not be definitively assigned to α -granules versus dense granules [11]. Indeed, one might well hold the platelet release reaction as a prime example of how multiple threads of research must come together in a definitive manner for a rational interpretation. We will return to the platelet release reaction later when we consider the route and mechanism of platelet granule release based on current available evidence.

α -Granule Structure/Function Relationships Meets Big Data and New Imaging Technology

Based on electron microscopy, protein localization and platelet activation studies, a general consensus on α -granule structure and organization had formed by late the 1990s to early 2000s [12–14]. In brief, α -granules in resting, circulating platelets are at a first approximation defined by their morphology: round to ovoid, large (200–500 nm), and abundant (~50–80 per cell). In contrast, mitochondria (aka β -granulomes) or dense granules (aka δ -granulomes) were present in fewer copies per platelet. α -Granules are further distinguished by a number of features: 1) a limiting membrane containing p-selectin as a relatively specific, membrane marker protein; 2) an electron dense, luminal matrix, a feature common to many protein storage granules in which the stored or cargo proteins are condensed; 3) a particularly electron dense nucleoid thought to be rich in proteoglycans (Figure 2A); 4) a peripheral cluster of 12–20 nm diameter tubules corresponding to multimeric oligomers of von Willebrand factor (VWF); and 5) the presence of small vesicles (potential exosomes, 40–100 nm in diameter). Occasional tubular structures with α -granule

like matrix were also observed. Whether these were a separate class of organelle was not clear from the thin sections. In sum, the consensus of the early 2000s was that α -granules in circulating platelets consisted of a single major population with at most minor variations.

A major conceptual challenges to this unitary model of α -granule organization and structure came almost immediately with the advent of big data approaches, namely, proteomics and the emergence of new imaging approaches which are addressed in the next section of this review. As revealed repeatedly through mass spectrometry, platelet α -granules contain and release hundreds of proteins [15–16; see Table 1] many of which have apparently contradictory physiological function, e.g, pro-versus antiangiogenic factors [17]. This has led to the repeated suggestion that there ought to be multiple subtypes of α -granules, which would contain distinct, physiologically significant cargo sets [17–18]. If true, the granule differences then may well be molecular with no differences in structure or α -granule organization. This point will be addressed further under the heading of “A Current Status Report”. For a detailed consideration of proteomics, the reader is referred to a review by in this issue of the journal.

A second major challenge to this model comes from the advent of new imaging technologies. These center on three advances: super resolution light microscopy, the first light microscope method that gives resolution appropriate for mapping proteins to structures of the size α -granules; better dehydration techniques for electron microscopy that appear to call into question features such as the α -granule nucleoid, and the arrival of practical whole platelet, 3-dimensional electron microscopy techniques. Whether these advances will provide a new outcomes that simplify interpretation or ones that will require some years to digest and reach consensus remains an open question. These possibilities are discussed below.

New Standards for Imaging Platelets

Super resolution fluorescence microscopy reduces the resolution barrier for rapid mapping of protein distribution within platelets

Conceptually, immunofluorescence microscopy should provide a rapid approach to mapping protein co-localization in platelets and addressing the question of the molecular homogeneity of α -granules. However, until recently the resolution of the optical systems relative to the dimensions of α -granules and their spatial separation within platelets was too limited to give more than a platelet-wide, spatial mapping of protein concentrations. There was no indication of the enclosing granule membrane and hence distributions could not be mapped relative to distinct individual granules. Direct measurement of granule dimensions from scanning transmission electron microscopy (STEM) image stacks give a physical measurement of the resolution needed. α -Granules are on the average have a diameter of 190 nm and a length of 340 nm and can be separated by distances as small as 20–50 nm. Hence, any microscopy approach should have a XY resolution better than 95 nm and a Z resolution of better than 170 nm to give any intragranular pattern information and even at that resolution granules will sometimes not be separated one from the other. Three-dimensional structured illumination microscopy (3D–SIM) can give that level of resolution and as illustrated in Figure 3 that resolution is sufficient to give much more granularity to

VWF or fibrinogen localization than older methods. 3D-SIM has already been used to show that VWF and fibrinogen appear to map to separate zones within a single larger structure [19], a result consistent with previous electron microscopy [20–21]. Despite a high instrument acquisition cost, ~\$750,000, 3D-SIM should become a wide spread approach in platelet granule biology. A second new fluorescence microscopy approach, dSTORM, has even better XY resolution, <50 nm, and has also begun to be applied to the platelets [22]. This is sufficient resolution to map protein distributions within individual α -granules.

In sum, with the commercialization of super resolution fluorescence microscopes capable of better than 100 nm lateral resolution, light microscopy today provides a set of tools for the examination of the internal organization of α -granules that are no longer significantly resolution limited as were older wide field and confocal microscopy approaches.

New electron microscopy sample preparation techniques and their possible implications for α -granules structure studies

The resolution of thin section electron microscopy is unparalleled, 1–2 nm in practice, and the limiting membrane of the α -granule and its matrix and other organelles can be readily revealed by heavy metal staining. Hence, electron microscopy has been a standard tool in granule structural biology. However, one must acknowledge that electron microscopy is dependent on sample preparation methods. Ideally, since platelets are in nature hydrated, structural biology of platelets should be done with hydrated samples. Technically, because the electron microscope operates at high vacuum to prevent the scattering of the electron beam by air molecules, hydrated sample must be viewed frozen in a cryo-chamber placed within the electron microscope column to preserve structural order. An important example of this is the recent work of van Nispen et al. [21]. They have plunge frozen platelets and then examined them by cryo-electron microscopy. Their work has revealed rare long tails as a feature of hydrated α -granules. Not surprisingly, such approaches are restricted to the thinner regions of the platelet due to electron scattering effects as sample thickness increases and by the extremely low contrast of the frozen, hydrated samples which are examined without the use of heavy metal stains. A second variation on frozen, hydrated samples is to take a frozen cryoprotected sample and either 1) section the sample as is done in typical immunogold labeling procedures in which the labeling and visualization is of thawed cryo-sections or 2) do the dehydration steps required for plastic embedding at a -90°C , a procedure referred to as freeze substitution. Platelets prepared by either of these frozen cell procedures display, in thin section, α -granules that are nearly devoid of nucleoids see Figure 2B [20, 23]. In the case of freeze substituted samples, the characteristic electron dense core seen in dense granules is preserved, an important control observation [23]. These results raise the open question of whether the α -granule nucleoid may be an artifact of sample preparation technique. In sum, sample preparation is still an aspect of electron microscopy that is subject to change and improvement as technology improves.

Three-dimensional electron microscopy to image full platelet volumes at high resolution

Until recently, electron microscopy has been a high-resolution tool with severe limitations in sampling volume. The typical 50 nm thin section samples approximately $1/50^{\text{th}}$ of the volume of a human platelet. However, this limitation can be largely to fully overcome with

3-dimensional electron microscope techniques. These consist of variations on either tilt-series electron tomography or serial-block-face scanning electron microscopy. In tilt series electron tomography, either standard beam geometry is used or STEM (scanning transmission electron microscopy) geometry [24–25]. In standard geometry, section thickness is limited to about 300 nm because electron scattering increases with section thickness. In STEM, an annulus is used to limit scattered electron detection and sections as thick as 1500 nm, 1.5 μm , can be used [23]. In serial-block-face microscopy; 10 μm or greater depth can be imaged by progressively shaving away the sample block with either a chamber-enclosed microtome or a focused ion beam (FIB). The exposed block face is then repeatedly imaged. Each of these approaches has its pluses and minuses. For the moment, the tradeoffs are that as the volume sampled increases there is a loss in Z resolution. The tilt series tomography method has a best-case Z resolution of ~ 3 nm while the serial-block-face methods have a Z-step size of 20–30 nm. Tomography typically gives a XY resolution of ~ 3 nm, while serial-block-face approaches typically give 8–10 nm XY resolution see Table 2. This resolution could be improved to 4–5 nm at the cost of 4-fold increase in the size of the image stack (e.g. 16 Gb instead of 4 Gb). Serial-block-face SEM results for platelets are just beginning to be published and should be revealing [26]. Hence, it is too early to review the biological impact of these techniques.

A Current Status Report: Four Unresolved α -Granule Structure/Function Questions

In this section we review that status of four major questions about platelet granule structure and function. We will discuss what is known using the current state-of-the-art methods and what are the limitations of the data.

Are there one or multiple α -granule populations

This is a question that today centers on two main points. One is the question of whether or not there are multiple α -granule populations based on cargo protein content. In the simplest terms, do all α -granules contain the same set of cargo proteins or not? The other is what is the importance of minor structural classes of α -granule-like organelles. Are these to be considered as separate morphology-based classes of α -granules or “snapshots” of the steady state dynamics of an organelle population? We consider both of these points individually and then venture an opinion as to how consensus might be reached.

In practical terms α -granules contain hundreds of proteins, likely with some variation in content from granule to granule. It is perhaps easiest to first answer whether or not nearly 100% of α -granules are positive for major proteins; acknowledging that any answer will come from microscopy, not biochemistry. Microscopy is inherently an individual “incident” readout while biochemistry gives a readout that is averaged over a population. One must also acknowledge that qualitatively there may well be uniformity across the organelle population but variation in the relative amount of individual proteins present in each granule.

Quantitative variation might well reflect the “perfection or imperfection” of protein sorting during organelle formation. To date, the actual answer given in the literature has varied over time and has been dependent on the available technology of the time. For example, the

answer from wide field immunofluorescence microscopy, done before confocal microscopy, high resolution digital cameras, and pixel shift free filter sets, was that the tested proteins all look like they co-localize [6]. Later immunofluorescence studies with more precise technology revealed that major proteins such as VWF and fibrinogen did not co-localize at the pixel level suggesting that they might well be distributed separately [27–28]. However, these studies were done with no knowledge of the position of the enclosing organelle membrane and hence must be considered operationally as readouts of protein distribution rather than a granule readout per se. Electron microscopy gave precision and had earlier shown VWF to be eccentrically distributed within individual granules [20]. However, because of limited sample volume in the thin sections, no conclusion could be reached from electron microscopy as to whether or not all α -granules were positive for VWF. Later immunofluorescence using 3D-SIM, a super resolution light microscope technique also revealed VWF to be zonally packaged within α -granules [19].

Several laboratories have done multi-label, immunogold labeling of thawed, cryosections of resting or activated platelets [5, 28–31]. In single label experiments, all investigators come to the same answer, namely, that a large fraction of the α -granules are individually positive for the 3 to 4 markers used. In some cases, the marker proteins, platelet factor 4 (PF4) and fibrinogen, had a distinct zonal distribution within the granule while the final marker, β -thromboglobulin, was more generally distributed throughout the granule matrix [5]. In double label experiments, co-localization was seen sometimes [5] but not always [28]. Often these studies were qualitative and lacked quantification of incidence, labeling densities, etc. all studies were with thin sections and hence no more than about 25% of the granule volume was being sampled. Considering the non-homogeneous distributions of the immuno-specific label, the use of individual thin sections and the lack of quantification, there is no definitive answer as to what would be true over entire granule volumes. In brief, further work is needed in which results are quantified over full granule volumes, scored quantitatively for several platelets and compared with super resolution immunofluorescence results in which several different granule cargo proteins can now be mapped rapidly relative to an α -granule membrane protein marker. Such a definitive quantitative answer will hopefully emerge within the next few years.

In closing this section, we briefly note that recent 3D electron microscopy studies by van Nispen et al. [21] and Pokrovskaya et al. [23] have all provided evidence for rare α -granule-like examples that are very elongate compared to the more common round to ovoid shape. Some have proposed that these are different class examples of α -granules while others have proposed that these are a reflection of α -granule dynamics, be they in secretion or granule-granule fusion in resting platelets. At present whether one or another answer or some combination of both are correct is not known. Recent work has shown that dense granules do undergo repeated kiss-and-run fusion [32]. Whether α -granules do the same remains an open question. The answer to whether there are different structural classes of α -granules is likely to emerge over the next few years through further 3D electron microscopy and detailed studies of α -granule dynamics.

What is the route of α -granule secretion

The best established model for secretory release is that of synaptic vesicles at nerve endings. In this case the vesicle is small, say 60–80 nm in diameter, and neurotransmitter release is accompanied by the vesicle fusing with the plasma membrane and vesicle membrane being absorbed into the plasma membrane [33]. In the platelet release reaction, α -granule contents are released from the platelet by a membrane fusion process. However, in contrast to the synaptic vesicle, the α -granule is not consumed in the process. In other words, an α -granule “ghost” remains behind with the membrane fusion being more a pore or opening that connects the granule ghost to the extracellular space. Moreover, pore formation may be a reversible event. Amperometric measurements of membrane capacitance show a “flickering” in capacitance values during the release reaction suggesting that pores are unstable [34–36]. The stability of the α -granule ghost membrane indicates that α -granule are not a major source of the membrane needed for pseudopodia formation during platelet activation and, in fact, platelets begin to form pseudopods before α -granule release.

In the case of the synaptic vesicle membrane, the vesicle fusion is with the plasma membrane of the synaptic ending. In the case of the α -granule, fusion has been proposed to be either directly with the plasma membrane or with the open canalicular system [OCS aka, surface-connected, canalicular system (SCCS) in older literature]. OCS appears as a series of enfoldings of the plasma membrane that greatly expand the surface area of the platelet. OCS was defined initially by the discovery that the enfolded network in thin section electron microscope experiments were coated with electron dense, membrane-impermeant mordants to selectively darken the membrane surface [37–41]. The evidence for α -granule fusion to the OCS then comes from the interpretation of mordant staining. Here the interpretation is made that decondensing α -granules that are mordant stained must by definition be connected to the plasma membrane by OCS even if the connection is short [29]. The evidence for direct fusion with the plasma membrane comes from non-mordant, electron microscopy observation and images showing decondensing α -granules fused to the plasma membrane by a short neck or longer continuity termed a pipe [23, 42]. In thin section, these continuities are relatively rare while in large volume techniques such as STEM they are more common and can be directly traced in 3-dimensional space. In conclusion, we raise the following as a future test of OCS involvement in α -granule release. The OCS in non-stimulated, washed platelets is highly interconnected. Hence, a possible validator of OCS mediated α -granule fusion would be networked connections of the apparent pipe to other OCS elements. In other words, is the connection, pipe, networked to other elements of the OCS. As 3D electron microscopy becomes more common, we propose that this should be a commonly used criteria.

What is the function of “closed” canalicular system elements

In essence, the canalicular system (CS) may be viewed as the holes in the swiss cheese. Structurally, in a thin section, electron micrograph, the CS is a series of membrane limited structures that are a few to several tenths of a micron in diameter and have no obvious electron dense matrix in their interior (Figure 2). Although much of this system is connected to the plasma membrane in human platelets immediately fixed upon blood draw and hence deservedly referred to as OCS, recent large volume, 3D electron microscopy reveals that a

significant of what would upon simple structural examination be referred to as OCS is, in fact, closed with no connection to the cell surface [23]. As shown by Pokrovskaya et al. [23] the CS appears to be dynamic with almost half being closed in resting platelets that were fixed immediately. Only a small number are closed in activated platelets and indeed, the entire CS become more interconnected with activation with the extent of fusion with the plasma membrane increasing significantly. No interconnections between the CS and α -granules were observed. Thus the results from large volume, 3D electron microscopy place more emphasis on the dynamics of the CS than previous work and raise questions about the physiological function(s) of the closed “CS” elements. Yadav et al. [26] have suggested that some closed CS elements might well be the storage sites of Golgi glycosyltransferases and sugar nucleotides, proteins and small molecules that are secreted upon platelet activation [43–44]. The Golgi apparatus in circulating platelets may be structured about a set of what have previously been considered to be CS elements.

How do α -granules sense cell surface secretory signals

Platelets contain, at their plasma membrane, multiple receptor systems which allow them to respond to multiple agonists. Individual plasma membrane receptor systems could be coupled to individual responder systems at the granule level. Simply put, only some granules could respond to a given agonist, another variation on molecular heterogeneity. Despite, an initial publication, Ma et al. [45] reporting that PAR1 and PAR4 agonists counter-regulated endostatin versus VEGF release from human platelets, little subsequent evidence for thematic release presumably from different α -granule subtypes has been reported. Jonnalagadda et al. [46] report kinetic rather than thematic, context-sensitive differences in granule release in response to thrombin, PAR1, PAR4 and convulxin as agonist, a result inconsistent with α -granule subtypes. Consistent with these results, both Nguyen et al. [47] and van Holten et al. [48] report similar release patterns for PAR1 and PAR4 stimulation by mass spectrometry analysis. Finally, Velez et al. [49] find that almost all differences in platelet release following stimulation with thrombin versus collagen are a consequence of the proteolytic activity of the thrombin agonist on the releasate. Overall, the data indicate that α -granules respond as a single population to agonist stimulation suggesting that the intracellular input of any single agonist is sensed uniformly by the α -granule population.

Presumably, any intracellular second messenger generated by agonist stimulated cell receptors must be sensed by a granule associated protein exposed to the cytoplasm. To date most of the work on granule specific machinery proteins has focused on members of the SNARE family. These are proteins that mediate granule membrane fusion. Specifically, the membrane is marked by a v-SNARE for vesicle SNARE of which there are at least four in platelets: VAMP-2, -3, -7, and -8 [see article from Whiteheart laboratory in this issue for a detailed review]. Regulating the fusion properties of SNARE proteins is potentially one way to create a signal dependent response at the granule level. As shown by Peters et al. [50], VAMP-7 is associated with a set of vesicles identified as α -granules on the basis of TIMP2 and VEGF. These vesicles preferentially move to the platelet periphery during the substratum spreading of human platelets while VAMP-3 and VAMP-8 positive vesicles do not. The relationship of these events to any sensor mechanism is unknown. Whether these distinctions represent a previously unknown difference between α -granule subtypes remains

an open question. Without knowledge of the molecular mechanisms and the exact protein machines involved, it is difficult to know the full implications of the findings. In brief, more research is needed.

Emerging Evidence for Non-Conventional Platelet Protein Secretion

As suggested in the Introduction, the platelet is the source of unexpected protein secretion events that can involve more than the expected set of organelles. Here we cite some important illustrations of such surprises. Although the α -granule remains the major and best understood protein secretory organelle in platelets, it is an organelle that can still be found to contain unexpected proteins and to be important to unexpected processes in the circulation. For example, platelet α -granules have recently been shown to be the intracellular site for storage and subsequent release of VLK tyrosine kinase that acts extracellularly [51]. This finding solved a previously unsolvable paradox of extensive tyrosine phosphorylation of extracellular proteins in the absence of any known secreted protein tyrosine kinase. Somewhat along the same lines but now involving Golgi proteins, there is evidence for extracellular sugar modifications of proteins in the circulation which depending on reaction had either no obvious source of sugar nucleotide substrate or glycosyltransferase. Recent evidence points to the platelet as being the source of extracellular sugar nucleotide substrates [52] and glycosyltransferases [43]. In brief, evidence points to Golgi elements in the platelet as being the probable source of these substrates and enzymes [43]. Moreover, recent evidence suggests that Golgi elements in platelets may be organized to support selective secretion of glycosyltransferases or sugar nucleotide substrates [26]. Finally, we cite the case of protein disulfide isomerase, an endoplasmic reticulum resident protein, DTS in platelets, long known to be important to disulfide bond formation within the secretory pathway. Protein disulfide isomerase was shown almost 25 years ago to be released upon platelet activation [53] and over time to be important to thrombus formation [54]. Recent evidence points to protein disulfide isomerase being stored and secreted from T granules, a structure that may be a specialized subdomain of the DTS [55–56]. Indeed, the platelet and its protein secretory processes continue to present surprises and to be imperfectly understood for what now is approximately 60 years of research.

A Brief Perspective for the Future

In our opinion, structural studies will continue to play a major role in understanding platelet protein secretion and the organelles involved. We submit that the dynamics of α -granules in the resting platelet is an under studied area. Lysosomes and mitochondria in nucleated cells continuously fuse with one another in an organelle specific manner that is followed by subsequent reactions. Platelet granules may be continuously sensing the intracellular environment through such dynamics. There is certainly evidence for minor structural populations of α -granules. Whether these then represent separate α -granule subtypes remains an open question. They could also be dynamic intermediates in α -granule fusion/fission. Furthermore, α -granules may well display some heterogeneity in membrane fusion machinery. There is the example of VAMP-7 cited earlier. In our opinion both newer super resolution fluorescence microscopy techniques and 3D electron microscopy will go from emerging techniques to mainline techniques in platelet granule cell biology. As these are

both discovery techniques, their full impact over time is difficult to predict. A continuing and major challenge over time will be to relate the results of single cell work back to the in vivo situation in mice and humans. Emerging bioengineering models such as those being pioneered by Wilbur Lam [57] and the continuing efforts of people like Stalker and Brass [58] with the further development of in vivo models are apt to be important. As usual, the devil will be in the details and developing these into comprehensive frameworks that both encompass the science and support clinical practice. The tools are present today to resolve questions that have been present for some years and new ones that will emerge from using the new tools. In conclusion, we look forward to that happening and view this as a time to be hopeful for rapid progress. We note the recent review by Heijnen and van der Sluijs [59] on platelet granules and urge the reader to also give consideration to this article.

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Figure 1. Electron microscopy image of human platelets showing (A) immediate fixed and (B) washed platelets

Platelets in the blood stream are poised to activate. When immediately fixed upon blood draw as shown in A), the platelets are discoid in shape and typically appear elongated in electron micrographs of thin sectioned material [23]. Here examples of α -granules, putative canalicular system elements, dense granules, and dense tubular network are indicated by 1–3 letter abbreviations. In B), platelets drawn into citrate were purified in the presence of activation suppressor [43]. These platelets are referred to as “washed” platelets and such platelet preparations are a typical starting point for platelet release reaction studies. Note that washed platelets are more rounded and extend pseudopods. The α -granules have not released their contents. Scale bar = 500 nm.

Figure 2. Representative electron microscope images of human platelets prepared in two different ways (conventional dehydration (A) versus freeze substitution dehydration (B)) Arrows and arrowheads point to example α -granules within a human platelet. The α -granules in these thin section images are frequently round to ovoid and are approximately 200–300 nm in diameter/length. With conventional room temperature dehydration (A), the α -granules commonly display a relatively central electron dense staining area referred to as a nucleoid. Using the newer, state-of-the-art dehydration technique freeze substitution in which the fixed platelets are first exposed to organic solvent at -90°C , the platelet α -granules as shown in (B) still maintain an electron dense protein matrix. However, there is now no dense nucleoid suggesting that the nucleoid may be an artefact of dehydration. White arrows point to α -granules containing electron dense nucleoids in (A) and white arrowheads in (A) point to examples of condensation of the α -granule protein matrix away from the limiting granule membrane, another indication of possible artefact from the conventional dehydration procedure. Note that the α -granules are spaced apart within the human platelets at sufficiently small distances that the widefield or confocal fluorescence microscope will not be able to resolve the individual granules one from another (see legend to Figure 3 for more details). Scale bar = 500 nm.

Figure 3. Appearance of cargo protein stained α -granules in human platelets visualized by either confocal microscopy or 3D-structured illumination microscopy (SIM)

Images are MIPs, maximum intensity projections, that include all images in the 3D image stack. (A) Field of 6 human platelets imaged by confocal microscopy. The platelets are stained for fibrinogen (green) and VWF (red). These proteins are content/cargo markers for α -granules. There is little correspondence in the distribution of the two proteins and the number of stained puncta is small, ~15 for each protein. (B) A representative individual platelet stained for fibrinogen (green) and VWF (red) and imaged by 3D-SIM, a super resolution light microscopy technique. Again, there is little correspondence in the distribution of each of the two α -granule cargo markers. However, now the number of stained puncta is much larger, ~50–80 for each protein. The difference in number between the two approaches to fluorescence microscopy can be readily explained by the difference in resolution between the two techniques. Confocal microscopy like widefield microscopy is diffraction limited with a resolution of 235 nm for green light and 260 nm for red light. In comparison, the resolution of the 3D-SIM microscope is 2-fold better in XYZ, ~120 nm XY in simple terms. Considering the spacing of α -granules in human platelets, frequently <100 nm or so apart [Figure 2], confocal microscopy is then unable to resolve such adjacent granules from one another. The number of apparent granules by confocal or widefield fluorescence microscopy will be low. However, by 3D-SIM fluorescence microscopy, adjacent α -granules will frequently be resolved one from another because of the two-fold

better resolution and the number of apparent α -granules will be larger. Note that the distribution of fibrinogen or VWF fluorescence shown in these micrographs is a direct measure of the mass distribution of these proteins within the cell. It does not directly indicate the dimensions of any given α -granule. Scale bar = 2 μm .

Table 1 Listing of example known α -granule proteins

Membrane proteins	Abundance	References
Integral membrane/receptor/antigen proteins:		
lectin (CD62P)	***	[16, 46, 59, 60, 62]
GlycoproteinIIbIIIa (GPIIbIIIa/integrin α IIb β 3)		[60–62]
Glycoprotein Ib-IX (GPIb-IX)		[60]
Glycoprotein Iba (GPIba)		[62]
Glycoprotein IV (GPIV/CD36)		[60]
Platelet and endothelial cell adhesion molecule-1 (PECAM-1/CD31)		[15, 60]
Cluster of Differentiation 9 (CD9/p24)		[60]
Glucose transporter 3 GLUT-3		[16, 60]
Osteonectin (ON)		[60]
Erythrocyte band -7 (EB-7/Stomatin)		[16]
Trem-Like Transcript-1 (TLT-1)	*	[16]
Latent-transforming growth factor beta-binding protein 1 (LTBP-1)		[16]
Clusterin		[64–65]
Cluster of Differentiation 40 Ligand (CD40L)	**	[46]
Cluster of Differentiation 63 (CD63)		[20]
Major Histocompatibility Complex 1 (MHC 1)		[66]
GTP binding proteins:		
Granule Membrane Protein (GMP33)		[60, 67]
Ras-related protein Rab-4 (Rab-4)		[60]
Ras-related protein Rab-27B (Rab-27B)		[16]
Ras-proximate1 (Rap1)		[16, 60]
vSNAREs proteins:		
Vesicle-associated membrane protein-2 (VAMP-2)		[61–62]
Vesicle-associated membrane protein-2 (VAMP-3)		[61–62]
Vesicle-associated membrane protein-2 (VAMP-7)		[61–62]
Vesicle-associated membrane protein-2 (VAMP-8)		[61–62]
Matrix proteins	Abundance	References
Proteoglycan proteins:		
Histidine rich glycoprotein (HRGP)		[60]
Serglycin (SRGN)		[60–65]
Coagulation factors/cofactors/fibrinolytic proteins:		
Factor V		[60–62]
Factor VII		[60]
Factor XI		[60–61]
Factor XIII		[15, 60–61]
Kininogens		[60–61]
Protein S		[15, 60–61]

Membrane proteins	Abundance	References
Integral membrane/receptor/antigen proteins:		
		[19, 60, 62]
Adhesive proteins:		
		[16, 59, 61–62]
Fibronectin		[59–61]
Vitronectin		[59–61]
		[15–16, 59–62]
Thrombospondin		[59–61]
Cytokines/Chemokines:		
Growth related oncogene-alpha (GRO- α /CXCL1)	****	[46, 61]
	****	[15–17, 19, 46, 59, 62]
Epithelial Neutrophil-Activating Protein-78 (ENA-78/CXCL5)		[61]
Neutrophil Activating Peptide-2 (NAP-2/CXCL7)	****	[59–61]
Interleukin-8 (IL-8/CXCL8)	**	[46, 61]
Stemcell Derived Factor-1 α (SDF-1 α /CXCL12)	***	[46, 61–62]
Monocyte Chemoattractant Protein-1 (MCP-1/CCL2)		[61]
Macrophase Inflammatory Protein-1 α (MIP-1 α /CCL3)	**	[46, 61]
Regulated on activation, normal T cell expressed and secreted (RANTES/CCL5)	****	[46, 59, 61]
	**	[46]
Monocyte Chemoattractant Protein-3 (MCP3/CCL7)	*	[46]
Monocyte Chemoattractant Protein-2 (MCP2/CCL8)	*	[46]
Macrophase Inflammatory Protein-1 δ (MIP1 δ /CCL15)	***	[46]
Thymus and Activation Regulated Chemokine(TARC/ CCL17)	*	[46]
Interleukin-1 α (IL-1 α)	*	[46]
Interleukin-1 β (IL-1 β)	*	[46]
Oncostatin M (OSM)		
Angiogenic growth factors:	*	[17, 46, 61–62]
	**	[17, 46]
	***	[17, 61–62]
		[61–62]
	*	[46, 61]
		[61]
		[60]
		[62]
	****	[59]
Transforming growth factor beta (TGF- β)	*	[46]
	**	[46]
Anti-angiogenic factors:	***	[17, 61–62]
		[17, 61–62]
Proteases:	**	[46, 63]

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Membrane proteins	Abundance	References
Integral membrane/receptor/antigen proteins:		
Matrix metalloproteinase 2 (MMP2)	*	[46, 63]
Matrix metalloproteinase 9 (MMP9)	*	[46]
Tissue inhibitor of metalloproteinase 4 (TIMP4)		[60]
Platelet Derived Collagenase inhibitor (PDCI)		[60]
Plasminogen activator inhibitor 1 (PAI1)		[60]
Tissue factor plasminogen inhibitor (TFPI)		[15, 61]
Protease Nexin-II (PN-II)		
Necrotic factors:	**	[46, 63]
Tumor necrosis factor alpha (TNF α)	***	[46, 63]
Tumor necrosis factor beta (TNF β)		

***
Most abundant,

***
Abundant,

**
Less abundant,

Proangiogenic properties, Antiangiogenic properties

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Table 2

A comparison of light and electron microscope imaging techniques

Imaging technique	Resolution	Strengths	Weaknesses	References
Widefield [Detection methods: bright field, dark field, phase contrast, differential interference contrast (DIC), fluorescence]	~230 nm in XY and ~800 nm in Z depending on wavelength (the longer the wavelength the poorer the resolution)	Multiple detection modes, Easy to operate, image is immediate with no need for calculation, both live and fixed specimens can be examined, fast multicolor image processing, Z-series data series collection less expensive, haze can be removed by deconvolution	All optical techniques lose resolution as sample becomes thicker, out-of-focus light haze can severely limit resolution	[68–70]
Confocal (Detection methods: fluorescence, DIC)	~230 nm in XY and ~500 in Z depending on wavelength	Improved Z resolution, elimination of out-of-focus haze, both live and fixed specimens can be examined, Z-series data collection.	Typically laser-based excitation and hence excitation limited to that of available laser set	[68, 70]
Super resolution:3D-Structured illumination Microscopy (3D-SIM) (Detection methods: fluorescence)	~100–120 nm in XY and ~200–300 nm in Z dimension	Capable of multicolor 3D imaging with existing fluorescence dyes, 2-fold axial and lateral resolution, total= 8-fold in 3D space.	Artifacts can be produced a low light levels during reconstruction of image	[22]
Direct stochastic optical reconstruction microscopy (dSTORM) (Detection methods: fluorescence)	~20–30 nm in XY and ~50–80 nm in Z dimension	Capable of analyzing at single molecule level, protein localization to small structures with high resolution, 3D capability.	Requires high mechanical stability microscope, images accumulated over many frames (40,000 frames per final image)	[22, 71–72]
Electron Microscopy (EM): Detects differences in electron density Thin section (~50 nm): Straight morphology/ protein localization in plastic sections Cryo immunogold	1–2 nm in XY	Metal stains to give differential contrast in electron density, can be used to identify different antigens present in tissue/specimen Label specimen in near native environment, sensitive method for immunodetection of antigen, serial sections can give 3D depth	Serial sections required to give 3D depth, requires pre-embedding extraction of cells/ tissue to achieve antibody penetration throughout cell/ tissue	[73] [74–76]
Electron tomography ~200–300 nm semi-thick plastic sections	~3 nm XYZ resolution	Provide 3D information from 2D projections, as data are digital objects can be viewed from any direction	Comparatively time consuming, requires intermediate voltage electron microscope (EM), insufficient depth to view full platelet volume	[77]
Scanning transmission electron microscopy (STEM) ~1000–1500 nm thick plastic sections	~5 nm XYZ resolution	Can provide near full platelet depth information at comparatively high resolution	Insufficient depth to view full platelet cell volume, requires intermediate voltage EM with STEM	[23–24, 78]
Plunge frozen cells Up to 500 nm sample depth	~4–5 nm	High resolution imaging under native sample hydration conditions	Very low contrasts, sample damage by electron beam can be a problem	[21, 79–80]
Focused ion beam-scanning electron microscopy (FIB-SEM)	~5–10 nm in XY ~20–30 nm in Z	Readily provides full platelet or megakaryocyte depth imaging of stained, high contrast samples embedded in plastic	Time consuming image collection, Image file sizes become very large (>10 Gbytes) as either pixel size is decreased to give greater XY resolution or greater depth is achieved	[79, 81]

Imaging technique	Resolution	Strengths	Weaknesses	References
<i>Serial block face-scanning electron microscopy (SBF-SEM)</i>	~5–10 nm in XY ~30 nm in Z	Readily provides full platelet or megakaryocyte depth imaging of stained, high contrast samples embedded in plastic	Time consuming image collection, Image file sizes become very large (>10 Gbytes) as either pixel size is decreased to give greater XY resolution or greater depth is achieved	[82–84]