Glycoconj J (2015) 32:9–15
DOI 10.1007/s10719-015-9573-y

MINI-REVIEW

Sulfated glycans in sea urchin fertilization

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Received: 13 December 2014 / Revised: 7 January 2015 / Accepted: 8 January 2015 / Published online: 31 January 2015

Abstract Fertilization is a controlled cell-cell interaction event that ultimately leads to the union of the gametes involved in reproduction. Fertilization is characterized by three major steps: (i) sperm binding to the extracellular matrix that coats the egg, inducing thereby the acrosome reaction; (ii) penetration of the acrosome-reacted sperm through the egg coat until its contact with the egg plasma membrane; and (iii) adhesion and fusion of the cell membranes of both gametes and the interchange of genetic materials. The acrosome reaction in the first step is important because it ensures that fertilization occurs only between gametes of homologous species. This specificity is primarily driven by the structure of egg jelly coat glycans recognized by a lectin-like binding protein (receptor) in the sperm membrane. Sea urchin fertilization is the best model utilized for understanding carbohydrate-mediated acrosome reactions. This report aims at describing the biochemical basis of regulatory mechanisms exerted by sea urchin sulfated fucans and galactans of well-defined chemical structures on the egg-sperm recognition process during fertilization of this invertebrate. Flagellasialin, a sulfated polysialic acid-containing glycoprotein found in sea urchin sperm flagella, is another sulfated glycan example also involved in fertilization of the echinoderm.

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Keywords Acrosome reaction · Fertilization · Sea urchin · Sulfated galactan · Sulfated fucan

Abbreviations

AR	Acrosome reaction
ECM	Extracellular matrix
Fuc*p*	Fucopyranose
Gal*p*	Galactopyranose
GPI	Glycosylphosphatidylinositol
Neu5Ac	*N*-acetylneuraminic acid
SG	Sulfated galactan
SF	Sulfated fucan
suREJ	Sea urchin receptor for egg jelly

Introduction

Fertilization is the initial event that occurs during the generation of offspring in multicellular organisms of sexual reproduction. It comprises the very first cell-cell interaction in life of the new organism, in which the gamete (sperm) of male ancestor encounters, binds and reacts with the gamete (egg or oocyte) of the female ancestor. Fertilization is a well-controlled biological process, in which structural components embedded in the extracellular matrix (ECM) of female gamete play a crucial role to ensure that only specific sperm can bind and react with the egg. For instance, rapid and efficient egg-sperm recognition and binding are imperative to aquatic animals of external fertilization, especially those living with closely related species at the same ecological niche like sea urchins [1].

In addition, sea urchins comprise the best models utilized for *in vivo* and *in vitro* studies about fertilization and developmental biology [2–5]. This is likely because of multiple factors such as (i) intense gametogenesis - both

female and male sea urchins release naturally and experimentally reasonable amounts of gametes in any annual season; (ii) easy recognition of the gamete genders - while male gametes are fairly white, female gametes are usually pinkish or yellowish; (iii) the biochemical events and components involved in the sea urchin fertilization are well-described [5]; and (iv) the structures of the egg jelly coat glycans involved in the regulation of the egg-sperm recognition process are very unique [6–8].

Figure 1 illustrates the major components and events involved during the sea urchin fertilization. The major components of sea urchin sperm are located inside the head, and comprise (i) the acrosome, an organelle which resembles the lysossome because it contains several digestive enzymes; (ii) the actin, a globular protein that helps forming microfilaments on the cytoskeleton and undergoes polymerization after positive egg-sperm recognition forming thus the acrosomal process; and (iii) the genetic material-containing nucleus responsible to transmit the genotype characteristics across generations.

The major components of the egg (Fig. 1) are the following: (i) the surrounding jelly coat which comprises a dense ECM composed of multiple functional molecules such as sulfated glycans, sialoglycans and peptides; (ii) a vitelline layer located right below the egg jelly, named *zona pellucida* in mammal ovum, and that contains mostly protein fibers; (iii) a perivitteline space located right between the vitelline layer and the oocyte plasma membrane; and (iv) the cortical granules located inside the egg cytoplasma at the periphery of the cell. The two latter components participate in the slow blocking process to avoid polyspermy.

The controlling mechanisms in sea urchin fertilization

Carbohydrate-based mechanism

When the sea urchin sperm approaches the egg of a homologous species (Fig. 2a), the first controlling mechanism involved in the egg-sperm interaction process is driven by a carbohydrate-protein interaction event [1, 3]. In this event, a multidomain 210 kDa protein receptor, named sea urchin Receptor for Egg Jelly (suREJ), located right above the acrosomal vesicle on the sperm surface recognizes sulfated glycans embedded in the jelly ECM of the sea urchin female gamete [5]. This carbohydrate-protein interaction triggers the acrosome reaction (AR). During the AR process, while hydrolytic enzymes are released from the acrosome vesicle to allow digestion of the dense egg jelly ECM coat, an acrosomal process consisted of polymerized actin is formed (Fig. 1). The conjugated and synchronized action of both degrading enzymes and the acrosomal protrusion helps the sperm to pass through the egg jelly coat and gain contact with the egg cell membrane.

Protein-based mechanism

On the tip of the acrosomal process of the reacted sperm, there is a protein named bindin [9] (Fig. 2b), which will participate in the next intermolecular recognition step of the egg-sperm interaction [7]. This step, driven by the bindin-receptor interaction, will trigger the ultimate reaction of both gametes, which is the fusion of their membranes to allow interchange of genetic information (Fig. 1). This protein-protein interaction event serves as

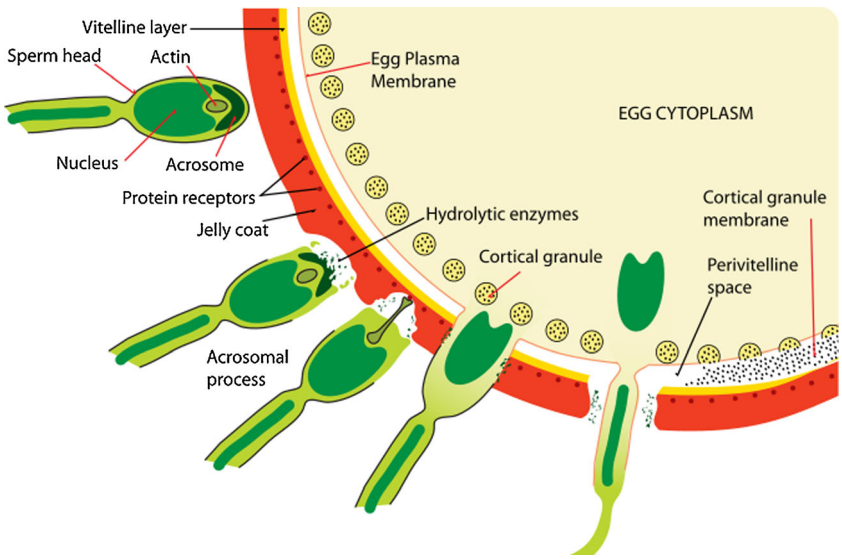

Fig. 1 Schematic representation of the major components and events involved during egg-sperm recognition in sea urchin fertilization (http://www.newworldencyclopedia.org/entry/sperm)

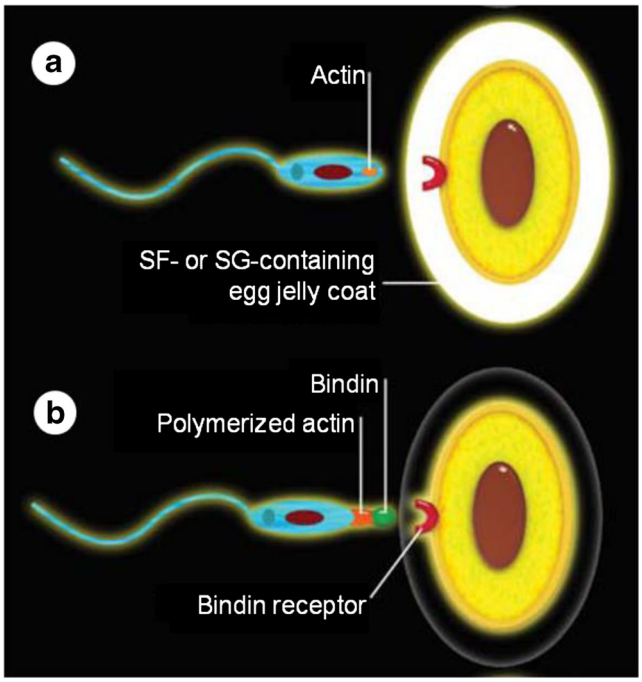

Fig. 2 Schematic representation of the two hierarchical regulatory events in egg-sperm recognition of sea urchin fertilization: the first one (**a**), a carbohydrate-based mechanism, is primarily driven by the sea urchin egg jelly sulfated glycans, sulfated fucan (SF) or sulfated galactan (SG), which play the role as inducers of the AR; the second one (**b**), a protein-based mechanism, driven by the bindin protein found at the tip of the sea urchin-reacted sperm, plays the role to ultimately enable cell membrane fusion of both gametes involved in the fertilization process

the final regulating mechanism to the species-specificity of the sea urchin fertilization as detailed below.

Egg jelly sulfated glycans

In order to keep the sea urchin AR (Figs. 1 and 2a) highly controlled in a manner that will safely ensure the species-specific fertilization - especially because this biological event occurs extracorporeally at the aquatic environment where closely related sea urchin species may co-habit - each sea urchin female species synthesizes a particular type of sulfated glycan (Fig. 3). These sulfated glycans, named sulfated fucans (SFs) or sulfated galactans (SGs), exhibit well-defined chemical structures for their regulative functions in sea urchin fertilization. SFs are polymers composed of α-L-fucopyranoses (Fuc*p*) (Figs. 3a-h). SGs are polymers composed of either α-L-galactopyranose (Gal*p*) (Fig. 3i), or β-D-Gal*p* units (Fig. 3j). While all structures are composed of oligosaccharide repeating units, the species-specificity varies in terms of certain structural features like sulfation patterns (but always 2-*O*- and/or 4-*O*-positioned); glycosidic linkages, α(1→3) (Fig. 3, panels A, B, D, E, G and H), α(1→4) (Fig. 3c and f), or β(1→3) (Fig. 3i and j); and the number of the composing residues within the oligosaccharide repetitive units: tetrasaccharides (Fig. 3a-c), trisaccharides (Fig. 3d), disaccharides (Fig. 3j), or monosaccharides (Fig. 3e-i), but they are all linear. The sulfate groups are highlighted by red ellipses in Fig. 3 for fast notation of the sulfation patterns. The SFs and SGs structures displayed in this figure are the following: (A) *Lytechinus variegatus* I [→3)-α-L-Fuc*p*-2,4(OSO_3^-)-(1→3)-α-L-Fuc*p*-2(OSO_3^-)-(1→3)-α-L-Fuc*p*-2(OSO_3^-)-(1→3)-α-L-Fuc*p*-4(OSO_3^-)-(1→]$_n$ [10]; (B) *Strongylocentrotus pallidus* [→3)-α-L-Fuc*p*-4(OSO_3^-)-(1→3)-α-L-Fuc*p*-4(OSO_3^-)-(1→3)-α-L-Fuc*p*-2(OSO_3^-)-(1→3)-α-L-Fuc*p*-2(OSO_3^-)-(1→]$_n$ [11]; (C) *Arbacia*

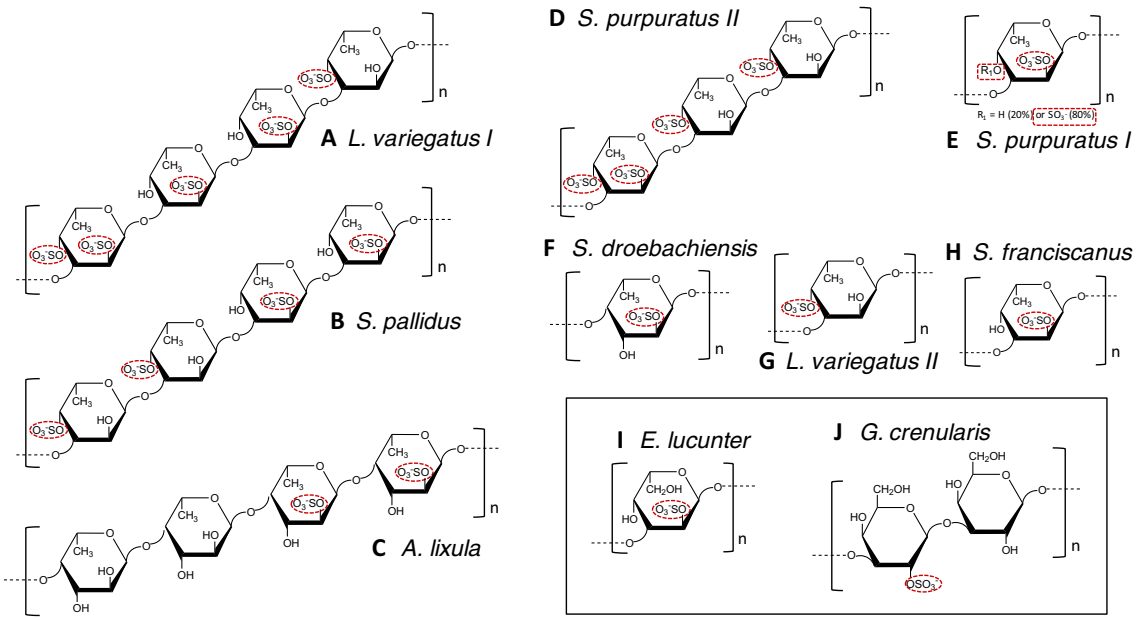

Fig. 3 Representation of the repetitive oligosaccharide units of sea urchin egg jelly sulfated fucans (**a**-**h**) and sulfated galactans (**i** and **j**, inside the *box*). Sulfation patterns are highlighted in *red*

lixula [→4)-α-L-Fuc*p*-2(OSO_3^-)-(1→4)-α-L-Fuc*p*-2(OSO_3^-)-(1→4)-α-L-Fuc*p*-(1→4)-α-L-Fuc*p*-(1→]$_n$ [12]; (D) *Strongylocentrotus purpuratus* II [→3)-α-L-Fuc*p*-2,4(OSO_3^-)-(1→3)-α-L-Fuc*p*-4(OSO_3^-)-(1→3)-α-L-Fuc*p*-4(OSO_3^-)-(1→]$_n$ [13]; (E) *S. purpuratus* I ~80 % [→3)-α-L-Fuc*p*-2,4(OSO_3^-)-(1→]$_n$ and ~20 % [→3)-α-L-Fuc*p*-2(OSO_3^-)-(1→]$_n$ [13]; (F) *Strongylocentrotus droebachiensis* [→4)-α-L-Fuc*p*-2(OSO_3^-)-(1→] $_n$ [11]; (G) *L. variegatus* II [→3)-α-L-Fuc*p*-4(OSO_3^-)-(1→]$_n$ [14]; (H) *Strongylocentrotus franciscanus* [3)-α-L-Fuc*p*-2(OSO_3^-)-(1→]$_n$ [15]; (I) *Echinometra lucunter* [→3-α-L-Gal*p*-2(OSO_3^-)-1→]$_n$ [12]; and (J) *Glyptocidaris crenularis* [→3-β-D-Gal*p*-2(OSO_3^-)-(1→3)-β-D-Gal*p*-1→]$_n$ [16]. The reasons that sulfated glycans from sea urchin egg jelly coats have well-defined chemical structures and that each species possesses its own particular structure suggest that these sulfated glycans may be involved in the species-specific regulation of the AR during the sea urchin fertilization.

Structure-function relationship in sea urchin fertilization

Sea urchin sperm AR can be experimentally measured *in vivo* through the incubation of isolated sperms with either purified sulfated glycans or the whole egg jelly. Positive reaction usually happens when elements from homologous sea urchin species are encountered. Table 1 shows the AR percentages obtained in assays using both homologous and heterologous sea urchin species. If we focus on the three species that co-habit the same aquatic niche in Rio de Janeiro, Brazil (*A. lixula*, *E. lucunter*, and *L. variegatus*), we can clearly see that their glycans are species-specific inducers of the AR in homologous species [12]. These sulfated glycans are inactive in heterologous species (Table 1). This is likely a result from the fact that the SFs from these three sea urchins are quite different from the structural point-of-view. While *L. variegatus* I has a 3-linked 2-*O*- and/or 4-*O*-sulfonated SF composed of pentasulfated tetrasaccharide repeating units (Fig. 3a), *A. lixula* expresses a 4-linked 2-*O*-sulfonated SF composed of disulfated tetrasaccharide repeating units (Fig. 3c), and *E. lucunter* synthesizes a 3-linked 2-*O*-sulfonated SG made up of monosulfated monosaccharide repeating units (Fig. 3i).

This species-specific induction of the AR, driven by egg jelly sulfated glycans of sea urchins who populate the same or at least spatially close marine environment, was further confirmed by the investigation on two *Srongylocentrotus* species from the North Pacific Ocean (*S. franciscanus* and *S. purpuratus*) [15, 17]. Both species express 3-linked SFs, but with different proportions of 2-*O*- and 4-*O*-sulfations (Fig. 3d and h). The sulfation patterns of these glycans are key players to control the AR induction in a species-specific way. As shown at Fig. 4, *S. purpuratus* sperms are quite sensitive to homologous 4-sulfated SF (red curve at bottom panel), but not to the heterologous 2-sulfated SF (red curve at top panel). Chemically oversulfation reaction on these glycans (blue sulfates at the structures of Fig. 4) did not change the positive response of the homologous SF, suggesting that increased 2-sulfation content does not alter the biological activity (blue curve at bottom panel of Fig. 4). Nonetheless, the SF from *S. franciscanus*, which was previously inactive on *S. purpuratus* sperm, becomes now active due to the increased 4-sulfation content (blue curve on top panel of Fig. 4). This oversulfated structure (top one at Fig. 4) is fairly equal to the active one shown at the bottom of Fig. 4. These datasets clearly indicate that while 2-sulfation is the structural player to control species-specific AR in *S. franciscanus*, 4-sulfation is the structural determinant for the *S. purpuratus* activity. Hence, in these two *Strongylocentrotus* species, the sulfation patterns of their SFs do determine the induction of the sperm AR, because their SFs have, despite the same major structural features like monosaccharide type (Fuc*p*), anomericity (α), glycosidic bond (3-linked), and enantiomericity (L), a distinct sulfation pattern.

The two above paragraphs have given reliable examples to clearly explain how the structural features of the sea urchin

Table 1 Percentage of AR assayed using sperms and sulfated glycans of different sea urchin species [12, 15–17, 22]

Glycan Sperm	*A.lixula*	*L. variegatus* I	*S.droebachiensis*	*S. purpuratus* II	*S.pallidus*	*S.franciscanus*	*E.lucunter*	*G.crenularis*
A. lixula	31	Nr						
L. variegatus	Nr [a]	29					Nr	
S. droebachiensis			51	1	13			
S. purpuratus			3	44	50			
S. pallidus			3	74	86			
S. franciscanus								
E. lucunter	Nr	Nr				32	65	14
G. crenularis								

Blank cells designate untested combinations

[a] Nr stands for non-reactive

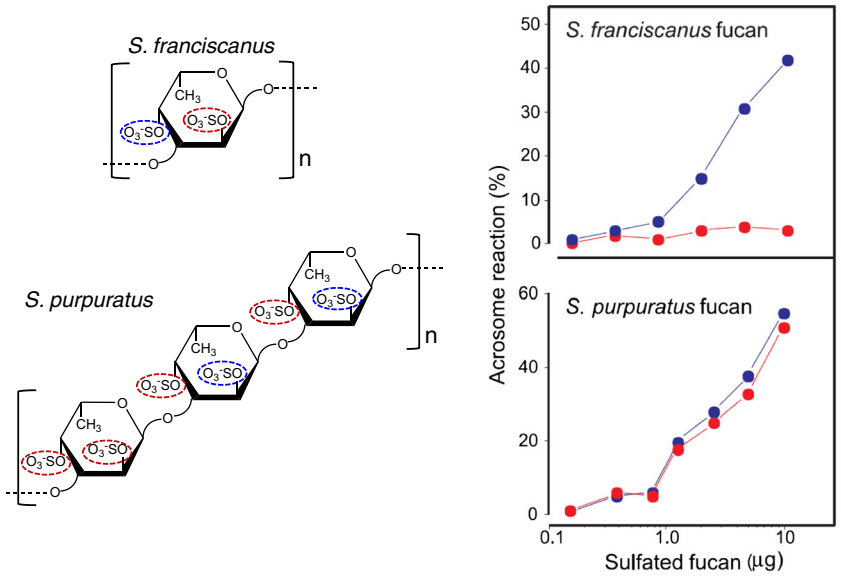

Fig. 4 Structures and effects of sea urchin egg jelly sulfated fucans of *S. purpuratus* II and *S. franciscanus* as inducers of the AR in *S. purpuratus* sperm. While red sulfates are original, blue ones are obtained by chemical oversulfation reaction [7]

egg sulfated glycans are able to trigger, in a well-controlled manner, the sperm AR across homologous species [12, 15, 17]. These sulfated glycans are acting in the first regulative step of the sea urchin fertilization (Fig. 2a). Nonetheless, when this first carbohydrate-based controlling mechanism is not itself sufficient to prevent the cross-reaction between different sea urchin species, the protein-based mechanism governed by the function of bindin [18–21] (Fig. 2b) takes place. Based on Table 1, cross-AR can be seen between the two *Strongylocentrotus* species *S. pallidus* and *S. purpuratus*. For instance, AR on sperm from *S. purpuratus* can be induced by heterologous *S. pallidus* 3-linked SF composed of two 2-*O*-sulfated and two 4-*O*-sulfated units within a tetrasaccharide repeating building block (Fig. 3b), in a potential very similar to the homologous SFs (Fig. 3d and e). The SFs from *S. purpuratus* are also highly-rich in 2-*O*- and 4-*O*-sulfations. The contrary assay works fine as well. AR in sperm from *S. pallidus* in the presence of the heterologous mixture of SF structures from *S. purpuratus* can be achieved with a potential nearly equal to that seen when the homologous *S. pallidus* SF is used (Table 1). Despite this positive cross-AR between these two sea urchin species, fertilized eggs of *S. purpuratus* were not achieved in a further experiment in which heterologous sperms, previously reacted with *S. pallidus* SF, were incubated with *S. purpuratus* eggs (in the presence or not of the *S. purpuratus* reacted sperm). Conversely, *S. purpuratus* reacted sperms alone were fairly able to fertilize the *S. purpuratus* eggs [22]. This set of results indicates that the second controlling mechanism regulated by bindin in the sea urchin fertilization (Fig. 2b) is the final regulatory event for impairing inter-speciation - especially in cases where sperm suREJ of one sea urchin species may be sensitive to the egg sulfated glycans of another species. Another interspecific AR was seen in the assay testing *E. lucunter* sperm with the heterologous SF from *S. franciscanus* [16]. This makes sense since the sulfated glycan structures of these two sea urchin species are very similar (Fig. 3h and i). Both are composed of 3-linked 2-sulfated α-L-units however, *E. lucunter* glycan is a SG whereas the one from *S. franciscanus* is a SF. The difference only at the monosaccharide type is not sufficient to prevent these species to cross-react in the AR assay. Further experiments to prove if *S. franciscanus* sperm-reacted is really not able to fertilize eggs from *E. lucunter* still remain to be performed.

One point that still remains to be discussed herein is why certain female sea urchins are able to synthesize more than one structure of sulfated glycans in their egg jelly coats, like the two SFs from *L.* variegates [14] (Fig. 3a and g), and the two SFs from *S. purpuratus* [13] (Fig. 3d and e). While structure I is active (Table 1), the structure II of *L. variegatus* is inactive in the AR in homologous sperms. Although the real reason for the synthesis of structure type II in *L. variegatus* is yet to be clarified, the fact that this structure is synthesized only at the winter raises speculations about the seasonal distribution of SFs in this sea urchin species and a possible role in the periodicity of the reproductive cycle of this invertebrate [14]. Conversely, both SF structures from *S. purpuratus* egg jelly are active in its homologous species [13]. The fact that *S. purpuratus* suREJ biochemically exhibits two carbohydrate recognition domains helps explanation on the AR sensitivity to both homologous SFs.

Biosynthesis of egg jelly sulfated glycans

Little is known about the biosynthesis of sulfated fucans and galactans. One of the few works concerning this topic has indicated that possible remodeling process, especially those

involving selective desulfation, may occur during the biosynthesis of these sulfated glycans [23]. Cinelli and associates have shown that a 6-desulfation occurred during the biosynthesis and maturation of the sulfated galactan from *E. lucunter* (Fig. 3i) may be the most reasonable explanation for the presence of a 2,6-di-sulfated 3-linked α-L-galactan in the oocytes of this echinoderm, despite the single presence of the monosulfated galactan in the egg jelly of this species. The authors speculated that the selective 6-desulfation is likely to be occurring when the eggs are spawned [23].

Sea urchin sperm flagellasialin

Sulfated fucans and galactans of the egg jelly coat in sea urchin (Fig. 3) are not the only sulfated glycans reported so far with action in the fertilization process of this marine invertebrate. In fact a novel α(2-9)-linked polysialic acid-containing glycoprotein of sea urchin sperm flagella has been identified and was named flagellasialin [24]. Flagellasialin from the sea urchin species *Hemicentrotus pulcherrimus* shows a diverse relative molecular mass, from approximately 40 to 80 kDa. Flagellasialin is composed of a 96-amino acid, threonine-rich protein core, heavily *O*-glycosylated. It was seen that flagellasialin can be highly expressed in the testis but not in the ovary of the echinoderm. The amino acid sequences of flagellasialin in three sea urchin species (*H. pulcherrimus*, *S. purpuratus*, and *S. franciscanus*) were compared and showed to be identical, although some species differences may exist in the three core glycan structures to which the sulfated α(2-9)-linked polysialic acid chain, composed mostly of *N*-acetylneuraminic acid (Neu5Ac), is linked. The treatment of sperm with a specific antibody against the α(2-9)-linked polyNeu5Ac structure resulted in elevation of intracellular Ca^{2+} and inhibition of sperm motility and fertilization. This has indicated that flagellasialin is acting as a critical regulator of these processes [24]. Recent data have confirmed the activity of sea urchin sperm flagellasialin in intracellular Ca^{2+} regulation of the sea urchin sperm, especially in events related with fertilization [25]. A more detailed investigation concerning the structure of flagellasialin has shown that this molecule is a glycosylphosphatidylinositol (GPI)-anchored glycoprotein highly glycosylated with the sulfated α(2-9)-linked polyNeu5Ac chains [25].

Conclusions

Fertilization is a well-regulated biological process which ultimately leads to the union of the gametes involved in a reproduction event. Fertilization is generally characterized by multiple sequential steps, in which the major ones are: sperm binding to the egg ECM resulting in AR induction; penetration of the sperm-reacted through the dense egg ECM till its contact with the egg plasma membrane; and finally, adhesion and fusion of cell membranes of both gametes in order to allow interchange of genetic materials. The species-specificity of AR in many metazoans [26], including marine invertebrates of sexual reproduction such as deuterostomes like the echinoderms sea urchins, is primarily controlled by carbohydrates coating the egg cell.

In sea urchin fertilization, this carbohydrate-driven species-specificity of AR occurred during the egg-sperm recognition is primarily regulated by the structural features, especially sulfation patterns, of the egg jelly coat sulfated glycans named sulfated fucan and galactans. Sea urchin sulfated fucans and galactans have structures characterized by well-defined repeating oligosaccharide units. In sea urchin fertilization, when the carbohydrate-driven controlling mechanism does not function for properly blocking the inter-specific interaction, a second regulating mechanism, governed by the protein bindin, takes place before the ultimate fusion of the gamete plasma membranes. This bindin-driven event guarantees at a last moment that fertilization does not occur across sea urchins of different species. Flagellasialin, a highly glycosylated GPI-anchored glycoprotein containing sulfated α(2-9)-linked polyNeu5Ac glycan, found in sea urchin sperm flagella is another example of sulfated compound involved in fertilization of the echinoderm. This molecule seems to be involved in the intracellular Ca^{2+} regulation. This review has comprehensively revisited the biochemical steps as well as the functional and structural properties of these sulfated glycans involved in sea urchin fertilization.

Future perspectives in studies concerning these molecules would be (i) a more detailed investigation about the biosynthesis of these molecules, either at the female gonad, ovary sulfated fucans and galactans, or at the male gonad, testis flagellasialin; (ii) the conformational and dynamical behaviors of these sulfated molecules both free in solution and (iii) bound to their functional ligands related with fertilization.

The more detailed investigation about the biosynthesis of sulfated fucans, galactans and flagellasialin comprise a totally new avenue of research in the field of glycobiology. This subject may comprise the report of genes involved in the synthesis of these sulfated glycans as well as the recognition and notation of the related enzymes such as glycosyltransferases and sulfotransferases. Sulfatases may be also involved in the process, as indicated with the work [23].

The conformational and dynamical analyses of these sulfated glycans free in solution or bound to their functional partners involved in the processes of fertilization will surely lead to a robust background concerning how these molecules may be working in this particular biological action. Structural features involved in the biological events can be also identified based on this type of investigation. Recently, we have initiated a study on this matter [27]. We have observed that sulfation patterns, although being the major structural

determinants in biological events such as AR, are not the ultimate regulators. In fact, the dynamical and conformational properties, which are in turn directly regulated by sulfation patterns, are the ultimate regulators [27]. Together with investigations concerning the biosynthesis of these relatively new sulfated glycans, the area of structural biology comprises also a fertile path in the science of these molecules.

Acknowledgments This study was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) grant Universal-14/2013-[470330/2013-9] and Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) Grant E-26/110.961/2013. I acknowledge Dr. Noritaka Hirohashi, Prof. Ana Cristina Vilela-Silva, Prof. Victor Vacquier and Prof. Paulo Mourão as well as their group members who have contributed to the major achievements revisited here. I apologize for those contributors and their works which were omitted.

Conflict of interest The content of this work is solely the responsibility of the author and does not necessarily represent the official views of the funding agencies. The author states that he is not aware of any authorship, affiliations, memberships, funding, or financial holdings that might be perceived as damaged or as affected by the objectivity of the content of this material. The author declares no conflict of interest by any part. The permission to reprint or modify published figures was obtained under copyright and license agreement.

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