A carbohydrate-based mechanism of species recognition in sea urchin fertilization*

Abstract

In the present review, we describe a systematic study of the sulfated polysaccharides from marine invertebrates, which led to the discovery of a carbohydrate-based mechanism of sperm-egg recognition during sea urchin fertilization. We have described unique polymers present in these organisms, especially sulfated fucose-rich compounds found in the egg jelly coat of sea urchins. The polysaccharides have simple, linear structures consisting of repeating units of oligosaccharides. They differ among the various species of sea urchins in specific patterns of sulfation and/or position of the glycosidic linkage within their repeating units. These polysaccharides show species specificity in inducing the acrosome reaction in sea urchin sperm, providing a clear-cut example of a signal transduction event regulated by sulfated polysaccharides. This distinct carbohydrate-mediated mechanism of sperm-egg recognition coexists with the bindin-protein system. Possibly, the genes involved in the biosynthesis of these sulfated fucans did not evolve in concordance with evolutionary distance but underwent a dramatic change near the tip of the Strongylocentrotid tree. Overall, we established a direct causal link between the molecular structure of a sulfated polysaccharide and a cellular physiological event - the induction of the sperm acrosome reaction in sea urchins. Small structural changes modulate an entire system of sperm-egg recognition and species-specific fertilization in sea urchins. We demonstrated that sulfated polysaccharides - in addition to their known function in cell proliferation, development, coagulation, and viral infection - mediate fertilization, and respond to evolutionary mechanisms that lead to species diversity.

Key words
- Acrosome reaction
- Fertilization
- Sulfated fucans
- Glycosaminoglycans
- Sulfated galactans
- Species diversity
- Sperm-egg recognition

Received September 18, 2006
Accepted October 31, 2006

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*This review is dedicated to Prof. Carl Peter Dietrich, in memoriam.

Research supported by CNPq and FAPERJ

A preamble: memories of the beginning at Prof. C.P. Dietrich’s laboratory

This review is dedicated to the memory of Prof. C.P. Dietrich, who died on February 1, 2005 in São Paulo. I started my scientific career in 1971 under the supervision of Prof. Dietrich while I was a medical student at Escola Paulista de Medicina (presently Federal University of São Paulo). As I look back on this period of my life, two major impacts related to my involvement with Prof. Dietrich’s group come to mind. The first was
the conviction he transferred to all his students that we were involved in international and competitive science although working in a poor and less developed country. My emphasis on this aspect may surprise most of us, the Brazilian readers, because the extraordinary development and growth of science in our country during the last three decades. But the general spirit at that time was that “we do not produce new knowledge but just repeat what has already been done in the developed nations”, as some of my friends and even some professors at Escola Paulista de Medicina used to say. Certainly it was a generation of Brazilian scientists that helped place Brazilian science on its present professional level, and Prof. Dietrich was an important leader of that generation.

The second impact on my memory comes as Prof. Dietrich participated in a symposium in Argentina in honor of Prof. L. Leloir, who received the Nobel Prize due to his work on biosynthesis of carbohydrates. Prof. Dietrich was impressed by the opening lecture of Prof. Leloir, when he mentioned that the success of a soccer player is to position himself properly in the field and then wait for the ball. If he tries to follow the ball he will always arrive late. Curiously, this is the same advice given by Pelé, the famous Brazilian soccer player. Prof. Leloir extrapolated the philosophy of the soccer game to the scientific activity of young scientists. He suggested that they should direct their work based mostly on their own results instead of following the literature. When a scientific paper is published, the author is already forward in his experiments, and if we try to follow his line of work we will tend to arrive last. Look for new ideas in your own results, advised Prof. Leloir, and create your way in science with imagination and creativity. I kept these comments in mind as the “Leloir/Dietrich rule” and have made fundamental discoveries in our laboratory on the biological roles of sulfated polysaccharides.

Why were we interested in sulfated polysaccharides from marine invertebrates?

During my initial work in Prof. Dietrich’s laboratory I studied glycosaminoglycans from different types of cartilages. We reported that growth cartilages contain higher proportions of chondroitin 4-sulfate than articular cartilages, where chondroitin 6-sulfate is more abundant. Furthermore, we described a metabolic error characterized by a deficiency of 6-sulfation of chondroitin sulfate. These patients present pathological...
changes of their articular cartilages but without modifications of the growth cartilages (3,4). Overall, these observations indicate that chondroitin 4-sulfate is related to the ossification process while the 6-sulfated isomer is important for the structural integrity of the cartilages. Furthermore, the relative amount of chondroitin 4-sulfate increases in tumoral and arthrotic cartilages (5,6), in which calcification commonly occurs. In addition, phylogenetic studies have shown that cartilages from bony fish contain more chondroitin 4-sulfate than cartilages from cartilaginous fish (7). In other studies, Mathews and Glacov (8) showed that calcified skull cartilage contains more chondroitin 4-sulfate than uncalcified skull cartilage. The next step, from a phylogenetic point of view, was to look for cartilage-like tissues in invertebrates and to investigate the type of isomeric chondroitin sulfate they contain.

But we then faced a conceptual question regarding the definition of cartilage. What is in fact cartilage? We found a “definition” for cartilage in a classical book by Mathews (9): “Cartilage is like pornography. It is difficult to define but it can be easily recognized by a specialist”! However, we preferred a more formal concept of cartilage-like structures in invertebrates, which we defined as those with properties similar to vertebrate connective tissues, such as a structural function, a relatively high proportion of extracellular matrix and relatively low proportion of cells and, finally, a high concentration of proteoglycan or other acidic polysaccharides. With the help of some biology students we were able to identify two tissues in marine invertebrates which fulfilled these characteristics - the tunic of ascidians (Chordata - Tunicata) and the body wall of the sea cucumber (Echinodermata - Holothuroidea).

**Sulfated L-galactans in the tunic of ascidians**

The tunic of ascidians is an external supportive and protective skeleton (10). It consists of dense bundles of fibrillar material embedded in a loose network of fine fibrils, an organization reminiscent of the vertebrate cartilage.

Large amounts of sulfated polysaccharides were found in ascidian tunics. To our surprise, we found that this tissue does not contain glycosaminoglycans, as is the case for mammal connective tissues, but a unique sulfated galactan (11). The structure of this complex polysaccharide was determined using a vast array of techniques, such as periodate oxidation, methylation analysis and nuclear magnetic resonance spectroscopy (12-15). This polysaccharide consists mainly of a carbohydrate core of galactose glycosidically linked through position 1→4 and sulfated at position 3. In some species the central polysaccharide core is substituted at the O-2 position by non-sulfated galactose units. Even more significant, the sulfated galactans from ascidians are entirely constituted of L-galactose, the D-enantiomorph being entirely absent (16). This is unusual since D-galactose is the common constituent of glycoconjegates.

**A fucosylated chondroitin sulfate in the body wall of the sea cucumber**

The body wall of the sea cucumber is formed mainly by collagen fibers embedded in an amorphous matrix. Small irregular microfibrils which resemble the proteoglycans from mammalian tissues, form bridges between the collagen fibers (Figure 1A).

Again, in this tissue we did not find glycosaminoglycans with structures similar to those of mammalian tissues. The major polysaccharide in the sea cucumber body wall was denominated fucosylated chondroitin sulfate. It is composed of a chondroitin sulfate-like core containing side chains of sulfated fucose units linked to the glucuronic acid moieties through the O-3 position of the acid (Figure 1B). The pres-
ence of these unusual sulfated fucose branches obstructs the access of chondroitin sulfate lyases and hyaluronidases to the chondroitin sulfate core. However, after partial acid hydrolysis, which removes the sulfated fucose branches, the polymer is degraded by chondroitin sulfate lyases into 6-sulfated and non-sulfated disaccharides (18,19).

The biological relevance of these unusual polysaccharides found in marine invertebrates is unclear. Possibly, the presence of L-isomers of galactose in the ascidian polysaccharides and of sulfated fucose branches in the chondroitin sulfate from the sea cucumber makes these polysaccharides resistant to degradation by D-galactosidases, hyaluronidases and chondroitin sulfate lyases, and therefore prevents the digestion of the ascidian tunic and the body wall of the sea cucumber by microorganisms present in the marine environment. Especially noteworthy is also the observation that the polysaccharides from the marine invertebrates are in general more sulfated than the glycosaminoglycans from mammalian connective tissues. Perhaps interactions between components of the extracellular matrix in these organisms occur at higher salt concentrations than in vertebrates and therefore require polysaccharides with higher charge density.

Our initial comparative biochemical project, with a focus on a phylogenetic study of cartilaginous chondroitin sulfate, led us to an unexpected stage position. We had new sulfated polysaccharides with unique structures extracted from the invertebrate tissues. Of course these findings opened new perspectives, such as studies on the biosynthesis of these unique polysaccharides (20,21), and an interest in their possible effects as modulators of a variety of events, such as coagulation (22-25), thrombosis (26-28), proliferation of vascular endothelial and smooth muscle cells (29), angiogenesis (30), viral infection (31), etc.

Unique sulfated fucans in echinoderms

In addition to the fucosylated chondroitin sulfate, the body wall of the sea cucumber contains small amounts of a sulfated fucan. The purification of this polysaccharide was very laborious. But, once we overcame this challenge, the structure of the sulfated fucan was rapidly determined using a combination of methylation analysis and nuclear magnetic resonance spectroscopy (32). Again we were lucky to find a single polysaccharide, composed of tetrasaccharide repeating
A carbohydrate-based mechanism of species recognition units, in which the 4 residues are 3-linked fucose units differing only by specific patterns of sulfation at the O-2 and O-4 positions (Figure 1C).

Sulfated fucans have been found in other organisms, namely the cell walls of marine algae and the jelly coat of sea urchin eggs (for a review, see Refs. 33 and 34). The first isolation of sulfated fucans (also known as fucoidan) from marine algae was reported almost 90 years ago (35) but studies of their structure have led to controversial results. The first evidence showing that sulfated fucans also occur in sea urchin was published 58 years ago (36) but no structural study was performed on these compounds. We then decided to undertake a systematic analysis of the structure of the sulfated fucans from echinoderms and marine algae.

Our studies (23,24) and also studies from other laboratories (37,38) showed that the sulfated fucans from brown algae have complex and heterogeneous structures. More recent studies have revealed the occurrence of ordered repeat units in the sulfated fucans from some species of algae (33,34). Even in these cases, the presence of highly branched portions and the complex distributions of sulfate and acetyl groups highlight the heterogeneity of algal fucans. In contrast, the echinoderm polysaccharides have simple, ordered structures, which differ in the specific patterns of sulfation and/or position of the glycosidic linkages within their repeating units (39-42). Some of these structures are shown in Figure 2.

Sulfated fucans are species-specific inducers of the acrosome reaction during sea urchin fertilization

Sulfated fucans from marine brown algae and from the body wall of the sea cucumber are components of the extracellular matrix and have a structural function analogous to that of the proteoglycans from mammalian connective tissues. However, in sea urchins, the sulfated fucans are found on a gelatinous layer which surrounds the eggs. This specific localization of the sulfated fucans suggests that the polysaccharides may be involved in the fertilization of these invertebrates.

A necessary event for successful fertilization is the acrosome reaction. When sperm approach the sea urchin egg, the egg jelly induces the sperm acrosome reaction, which exposes the protein bindin at the tip of the sperm head. Only then can sperm attach to the egg and their plasma membrane fuse (43-46).

The acrosome reaction was discovered in sea urchins, starfish and several other marine invertebrates by J.C. Dan, working in Japan in the early 1950s. In 1947 J.C. Dan revisited her home country, the USA, for the first time after the difficult years in Japan during World War II. She took the first commercially available phase contrast microscope back to Japan and in the following year set up a research program on fertilization of marine invertebrates. Appreciating the great technological advantages of phase contrast microscopy, she first explored an old problem of sperm entry into eggs. Then she concentrated on the changes in the sperm tip upon fertilization, for which she also applied electron microscopy, another great technological innovation at that time. She discovered the biological significance of the changes, for which she introduced the term “acrosome reaction”, detected through careful observation of living spermatozoa under the phase microscope and intensive examination of spermatozoa, immediately after fixation in a sea water suspension, with an electron microscope. The electron microscope that she used was a very primitive model, although it was one of the latest in those days - the fourth machine of Hitachi Electron Micrograph. For a historical review, see Hoshi et al. (47).

Despite extensive studies on the morphological and physiological changes that
occur in the spermatozoa during the acrosome reaction in the following four decades, the specific molecule from the egg jelly involved in the induction of the acrosome reaction in sea urchins was not identified until 1994, when Keller and Vacquier (48) reported that “purified sulfated fucans had no significant acrosome reaction-inducing activity. Instead, acrosome reaction-inducing activity was associated only with two glycoproteins with approximate relative subunit masses of 82 and 138 kDa”. Even so, we decided to test whether sulfated polysaccharides from the sea urchin egg jelly induce the acrosome reaction. The well-defined chemical structure of these polysaccharides and the observation that each species possesses a polymer with a different structure (Figure 2) suggested that these sulfated polysaccharides may regulate a specific biological event during sea urchin fertilization. In fact, when we tested purified sulfated polysaccharides from the sea urchin egg jelly with homologous and heterologous sperm from species

Figure 2. Structures of sulfated polysaccharides from marine invertebrates. The figure shows 9 fully characterized structures of sulfated polysaccharides from the egg jelly of sea urchins and the tunic of ascidians. The specific pattern of sulfation and the position of the glycosidic linkage vary among sulfated fucans from different species. The structures of some α-L-galactans are also included in the figure. L-galactose and L-fucose are closely related sugars, differing only by the CH₂OH and CH₃ radicals at position 5, respectively. In fact, a common designation for L-fucose is 6-deoxy-L-galactose.
that co-habit the same area in Rio de Janeiro, we observed that the sulfated polysaccharides are species-specific inducers of the acrosome reaction (Figure 3).

The fact that the sulfated fucans are species-specific inducers of the acrosome reaction in sea urchins was confirmed when the study was extended to the species *Strongylocentrotus franciscanus* and *S. purpuratus*, both from the North Pacific Ocean (41,49). These species contain 3-linked fucans, which differ in the proportions of 2- and 4-sulfation. The pattern of sulfation is an important feature for recognition of fucans by the sperm. As exemplified in Figure 4, sperm from *S. purpuratus* were sensitive to homologous 4-sulfated fucan but not to heterologous 2-sulfated fucan (red symbols in Figure 4). In these 3-linked sulfated fucans the 2- and 4-positions are the only ones capable of sulfation. Oversulfation of the sulfated fucan from *S. purpuratus* did not change its responsiveness to homologous sperm, suggesting that increased 2-sulfates do not increase or inhibit the biological activity of this species (indicated by blue symbols in Figure 4). However, the sulfated fucan from *S. franciscanus*, which was ineffective on the *S. purpuratus* sperm, reacted as the homologous fucan after chemical oversulfation because of increased 4-sulfation. Thus, the position of sulfation is crucial for the acrosome reaction-inducing activity of the sulfated fucans of these *Strongylocentrotus* species rather
than the nonspecific charge density or sulfate content of the polysaccharides.

**Two mechanisms of sperm-egg recognition in sea urchin fertilization**

We demonstrated that sulfated fucans from the egg jelly induce the sperm acrosome reaction in a species-specific way. However, we had not investigated the contribution of this finding to the final biological event, that is, the fertilization of the sea urchin eggs. Furthermore, the relationship between the carbohydrate-based mechanism of sperm-egg recognition and the well-known bindin protein paradigm (43-46) remained to be clarified.

At this stage we outlined an experimental approach to distinguish between the bindin and the carbohydrate-based recognition mechanisms using three closely related sympatric *Strongylocentrotus* species (50). Initially we determined fertilization among the species assessed by counting the proportions of fertilized eggs (Figure 5A). For conspecific gametes we observed a high percentage of successful fertilization, whereas heterospecific sperm fertilized only a small percentage of eggs. After pre-reaction of the sperm with conspecific egg jelly, interspecific fertilization between *S. droebachiensis* and *S. pallidus* increased significantly in both directions. In contrast, the eggs of *S. purpuratus* could not be fertilized, even after reacting the heterologous sperm with conspecific egg jelly.

These observations indicated that induction of the acrosome reaction by the egg jelly sulfated polysaccharides is the major limitation for interspecific fertilization between *S. droebachiensis* and *S. pallidus* (carbohydrate-base species recognition mechanism). In contrast, the major limitation for interspecific fertilization between *S. purpuratus* and the other two species is a step that follows the acrosome reaction. Possibly this involves reaction of the bindin protein, exposed on the outside of the sperm tip, with the matching egg membrane receptor (the protein paradigm of species recognition). These two hierarchical steps in sea urchin gamete recognition are shown in Figure 6.

To investigate whether failure of the sperm acrosome reaction, induced by egg jelly sulfated polysaccharides, is in fact the major limitation of interspecific fertilization between *S. droebachiensis* and *S. pallidus*, we determined the proportion of sperm that undergoes the acrosome reaction in response to egg jelly sulfated polysaccharide (Figure 5B). *S. droebachiensis* sperm responded exclusively to the conspecific polysaccharide, whereas *S. pallidus* and *S. purpuratus* cross-reacted. *S. droebachiensis* contains a sulfated fucan with a structure unique among the other *Strongylocentroides* species examined, since it contains 4-linked instead of 3-linked fucose.

Figure 4. Structures and effect of sulfated fucans from the egg jelly of two *Strongylocentrotus* species as inducers of the acrosome reaction on homologous and heterologous sperm. These species contain 3-linked fucans, which differ in the proportion of 2- and 4-sulfation (represented in red). Chemical oversulfation of these polysaccharides adds additional sulfate esters to the polysaccharides at the 2- and 4-positions (represented in blue), the only ones capable of sulfation. Note that oversulfation of sulfated fucan conferred acrosome reaction activity to heterologous sperm but did not increase the activity of homologous ones. Data modified from Hirohashi et al. (49) (printed with permission from Elsevier).
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Figure 5. Experimental approach used to distinguish between the bindin and the carbohydrate-based mechanisms of sperm-egg recognition in sea urchin fertilization. A. Successful fertilization among three *Strongylocentrotus* species using sperm diluted in sea water (A-1) and pre-reacted with conspecific egg jelly (A-2). B. Structures of sulfated fucans isolated from the egg jellies of sea urchins and their effects as inducers of the acrosome reaction. The sulfated fucans were used at a concentration of 100 µg hexose/mL. Note that this is a higher concentration of sulfated polysaccharide than those used in the experiments in Figure 4. Egg jellies of *S. purpuratus* contain two isoforms of sulfated fucans shown in the panel. Data modified from Biermann et al. (50) (printed with permission from Blackwell Publishing). See text for further details.
units. This sulfated fucan specifically triggered the acrosome reaction only in sperm from its own species. Sperm from *S. pallidus* and *S. purpuratus* responded to conspecific and heterospecific 3-linked but not to 4-linked sulfated fucans, independent of their sulfation patterns at the O-2 and O-4 positions.

Overall, we have shown that sulfated carbohydrate from egg jelly induces the acrosome reaction species specifically in *S. droebachiensis* and *S. pallidus*. This characterizes a carbohydrate-based species recognition mechanism. There are no other significant barriers to interspecific fertilization between these two species. Other species pairs in the same genus acrosome react nonspecifically to egg jelly polysaccharide but exhibit species-specific sperm binding due to the bindin protein (the protein paradigm of species recognition) (Figure 6).

**Sulfated fucans: another avenue for speciation in sea urchins?**

Phylogenetic relationships, the times of divergence of sea urchin species and a summary of the structure of the polysaccharides from their egg jellies are shown in Figure 7. These observations indicate that the genes involved in the biosynthesis of the sulfated fucans did not evolve in concordance with evolutionary distance but underwent a dramatic change near the tip of the *Strongylocentrotid* tree driven by natural selection. Possibly, the acrosome reaction specificity may have played a direct role in the separa-

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**Figure 6.** Schematic representation of the two hierarchical steps in sea urchin gamete recognition. A. Carbohydrate-based species recognition: the sperm acrosome reaction is induced when a sperm with the correct receptor type contacts specific sulfated polysaccharide in the egg jelly coat (red triangles). This reaction exposes the bindin protein (shown in blue). B. The protein paradigm: the bindin protein, coating the outside of the sperm tip, reacts with a matching egg membrane receptor. AR = acrosome reaction. Reprinted from Biermann et al. (50) (with permission from Blackwell Publishing).

**Figure 7.** Phylogenetic relationship and divergence times of some sea urchin species and a summary of the structure of the polysaccharides from their egg jelly. Possibly, the carbohydrate-mediated mechanism of egg-sperm recognition played a direct role in the separation of *S. droebachiensis* from *S. pallidus*. The bindin mechanism may have functioned as an isolation mechanism on the earlier separation of the joint lineage from *S. purpuratus*. Myr = million years of evolutionary divergence. Data modified from Biermann et al. (50) (printed with permission from Blackwell Publishing).
tion of these species. There is evidence that *S. droebachiensis* and *S. pallidus* separated from *S. purpuratus* before their divergence from each other. The bindin mechanism may have functioned as an isolation mechanism in the earlier separation of the joint lineage from *S. purpuratus*. A renewed and later speciation event originated the species *S. droebachiensis* and *S. pallidus* due to the egg jelly sulfated fucans and sperm incompatibility (carbohydrate-based mechanism).

Surprisingly the egg jelly of the sea urchin *S. droebachiensis* contains a 4-linked sulfated fucan clearly distinct from those of the phylogenetically related species but similar to that from *A. lixula* - a species that diverged approximately 200 million years ago. This fact characterizes an event of convergent evolution - identical sulfated fucans in species that diverged millions of years ago. The occurrence of the same fucan in these two species is not relevant for their cross-fertilization because the populations of *A. lixula* and *S. droebachiensis* do not overlap geographically. Nevertheless, this observation suggests that the gene for the biosynthesis of 4-linked fucan may have been retained during the evolution of *S. droebachiensis*, but remained repressed or non-expressed until a period of strong natural selection. This observation reminds us of a general concept regarding evolution, stating that “the past of an organism not only determines its future but also gives an enormous reserve for a rapid modification, based on little genetic changes” (51).

**Conclusions and challenges**

Our studies on the structural characterization of sulfated polysaccharides from marine invertebrates led us to discover unique polymers in these organisms, especially sulfated fucose-rich compounds found in the egg jelly of sea urchins. These polysaccharides have simple, linear structures composed of repeating oligosaccharide units. They differ among the various species of sea urchins in terms of specific patterns of sulfation, the position of the glycosidic linkage within their repeating units and in the type of constituent monosaccharide. These polysaccharides show species specificity in inducing the acrosome reaction in sea urchins, providing a clear-cut example of a biological transduction event regulated by sulfated polysaccharide. This distinct carbohydrate-mediated mechanism for cell-cell recognition during fertilization co-exists with the bindin-protein system. Our results may lead to an overall view of carbohydrate-mediated fertilization in sea urchins and indicate how the egg jelly polysaccharide evolved and led to species diversity.

The major challenge at this stage is the identification of the metabolic pathways involved in the biosynthesis of the egg jelly polysaccharides. This is not only a fascinating challenge for carbohydrate researchers, but may also help to define the genetic basis for the proposed carbohydrate mechanism of species recognition.

Finally, we hope this review is convincing in the sense that we were able to obtain new results of general biological interest following the “Leloir/Dietrich advice”. The motive to follow this route was to look always for inspiration in our own results. We hope this is a proper way to honor the memory of Prof. Carl Peter Dietrich.

**Acknowledgments**

The contributions to this work by A.P. Alves, A.C.E.S. Vilela-Silva and A.P. Valente (Universidade Federal do Rio de Janeiro), B. Mulloy (National Institute for Biological Standards and Control), V.D. Vacquier (Scripps Institution of Oceanography) and C.H. Biermann (Friday Harbor Laboratories, University of Washington) are duly acknowledged. We are grateful to Dr. Maria Christina Mello (Universidade Federal do Rio de Janeiro) for revising the manuscript.
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