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Exploring the full potential of sperm function with nanotechnology tools

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Abstract

Sperm quality is essential to guarantee the success of assisted reproduction. However, selecting high-quality sperm and maintaining it during (cryo)preservation for high efficiency remains challenging in livestock reproduction. A comprehensive understanding of sperm biology allows for better assessment of sperm quality, which could replace conventional sperm analyses used today to predict fertility with low accuracy. Omics approaches have revealed numerous biomarkers associated with various sperm phenotypic traits such as quality, survival during storage, freezability, and fertility. At the same time, nanotechnology is emerging as a new biotechnology with high potential for use in preparing sperm intended to improve reproduction in livestock. The unique physicochemical properties of nanoparticles make them exciting tools for targeting (e.g., sperm damage and sexing) and non-targeting bioapplications. Recent advances in sperm biology have led to the discovery of numerous biomarkers, making it possible to target specific subpopulations of spermatozoa within the ejaculate. In this review, we explore potential biomarkers associated with sperm phenotypes and highlight the benefits of combining these biomarkers with nanoparticles to further improve sperm preparation and technology.

Keywords: biomarkers, functional genomics, nanoparticles, spermatozoa.

Introduction

Routine semen analysis, such as sperm motility and morphology, has been used as the main criteria for monitoring sperm quality and subsequent fertility. However, ejaculates approved by these criteria do not necessarily have high fertility (Avenidaño et al., 2009; Ferrigno et al., 2021), and there is a need for robust biomarkers to effectively predict fertility and reduce/prevent losses associated with infertility. Functional genomics, a technique capable of describing the functions and interactions between genes, proteins, and metabolites, has allowed the identification of several biomarkers associated with sperm phenotype, such as motility level, fresh, chilled, frozen, or fertility status. Numerous high-throughput technologies have been used to identify biomarkers related to sperm phenotypes, such as fertility and freezability (Peddinti et al., 2008; Soggiu et al., 2013; Menezes et al., 2019; Mateo-Otero et al., 2023; Song et al., 2023; Sun et al., 2023). These potential biomarkers offer novel perspectives for sperm preparations using nanoparticles.

Nanoparticles are nanoscale compounds produced naturally by cells as extracellular vesicles or manufactured through bottom-up (synthesis from atoms and molecules) and top-down (synthesis from bulk materials) approaches, possessing various physicochemical properties (e.g., electrical, optical, and magnetism). A typical structure of a nanoparticle with specific properties (e.g., magnetism and fluorescence), mainly acquired from its core composition is shown in Figure 1. The US National Nanotechnology Initiative describes nanotechnology as the understanding and controlling matter at the nanoscale, at dimensions between approximately 1 and 100 nanometers, where unique phenomena enable novel applications (NNI, 2024).

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Nanotechnology has sparked as one of the emerging research fields during the last decades, with bioapplications in human healthcare as diagnostic and therapeutic agents (Abedin et al., 2021; Anjum et al., 2021). This technology holds great potential in veterinary medicine and livestock farming in enhancing animal health, (re)production, and husbandry, acting as antimicrobials, animal growth and well-being promoters (nano-additives in feed), vaccines, and nanomaterials for drug delivery (Hill and Li, 2017; Selokar et al., 2020; ul Haq et al., 2023; Barwant et al., 2024). Especially in reproduction, nanotechnology has enormous potential in semen technology, as nanoparticles could be used for targeted sperm selection or nanoselection/nanopurification (Odhiambo et al., 2014; Feugang et al., 2015a), imaging, (Feugang et al., 2015b; Jain et al., 2018), sexing (Domínguez et al., 2018), and reduction of damages occurring during (cryo) preservation (Falchi et al., 2018a; Khodaei-Motlagh et al., 2022; Khaliq et al., 2023).

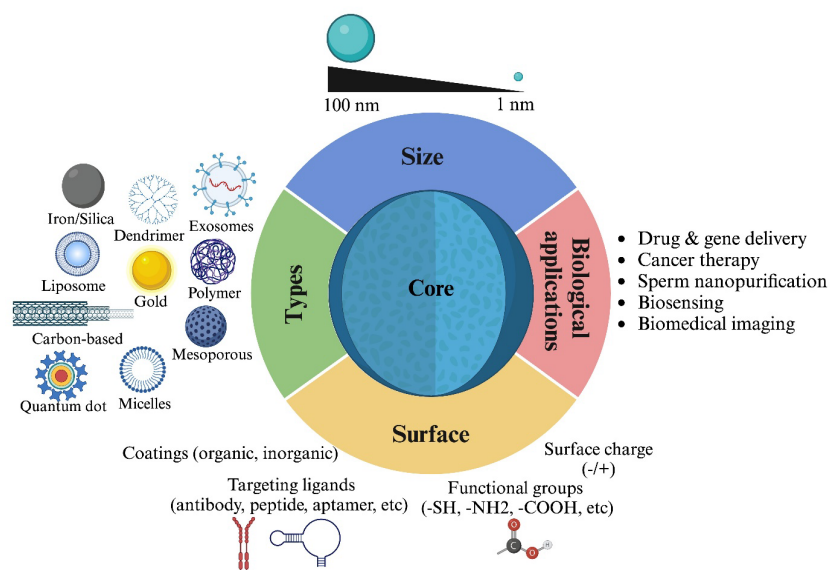


Figure 1. Structure of a typical nanoparticle. Metal nanoparticles (MNPs) have a metal core and a shell. The core can be made of an inorganic metal (zinc, iron, silver, gold, etc.) or metal oxide (Aluminum, copper, magnesium, titanium, zinc, silica, iron oxides, etc.). The shell can be made of an organic (e.g., polymers) or inorganic material (e.g., gold), or metal oxide (e.g., silane). Nanoparticles made of metallic cores surrounded by oxide shells, also known as metal oxide core-shell nanoparticles, have become popular due to their unique properties and high stability. Diverse metal-oxide interactions in metal oxide core-shell nanoparticles enable tuning their electronic structure (shape and size), spectroscopic properties, and surface reactivity for bioapplications (e.g., sensing, drug/gene delivery, and targeted imaging and selection).

From this background, semen has unique attributes that could improve the effectiveness of sperm biotechnologies. This review explores sperm biology and identifies key molecular markers linked to sperm phenotypes, such as motility, storage, freezing, sex, and fertility. It also highlights the benefits of combining these biomarkers with nanotechnology tools to improve sperm fertility and technological outcomes.

Sperm biology

Spermatogenesis

The spermatozoon consists of the head, which contains the genetic material (DNA); the middle piece, containing the mitochondria; and the tail, responsible for motility. Spermatozoa are highly specialized, compact, and motile cells originated after spermatogenesis to move to the ampulla region, where it interacts with the oocyte and initiate fertilization process. Spermatogenesis is a species-specific cell differentiation process that occurs in the seminiferous epithelial and is regulated by the hypothalamic–pituitary–testicular axis (Amann, 2008).

Spermatogenesis involves three distinct phases, namely proliferation (spermatocytogenesis), meiosis, and differentiation (spermiogenesis) (Staub and Johnson, 2018).

Proliferation phase or mitotic divisions – cell divisions occur in a sequence where chromosomes duplicate, resulting in two daughter cells or primary spermatocytes (or spermatocytes I), maintaining intercellular bridge connections with spermatogonia (types A and B). Spermatocytes I possess equal division of chromosomes (e.g. $2n=46$ and $4n$ centromeres) and cytoplasm (Sharma and Agarwal, 2011).

Meiotic phase – individual spermatocytes I move into the adluminal compartment, duplicate DNA, and divide into two haploid secondary spermatocytes (spermatocyte II, with $n=23$ chromosomes and $2n$ centromeres) (Alpatov et al., 2014). This second stage (meiosis II) immediately follows the first stage of the meiotic phase (meiosis I), involving chromosomal exchange with divided centromeres and giving rise to four haploid daughter cells ($n = 23$ chromosomes and 1 centromere) (Sharma and Agarwal, 2011).

Spermiogenesis – sperm cells undergo complete differentiation or morphogenesis to become highly specialized spermatozoa with compacted chromatin (Durairajanayagam et al., 2015). Numerous nuclear and cytoplasmic changes occur in spermatozoa during that phase. For example, nuclear histone is replaced with protamine to form well-developed disulfide bonds (Holstein et al., 2003), various organelles of the cytoplasm such as the Golgi apparatus, acrosomal cap, proximal centriole, and flagellum structures, go through profound remodeling and reorganization (Cooper, 2005). The midpiece surrounded by a sheath, axial core, and coarse fibrils (Neto et al., 2016) has a high concentration of mitochondria, which are responsible for aerobic functioning and supply energy for sperm motility (Gadella and Luna, 2014). After successive stages of differentiation, the resulting spermatozoa emerge as immature and immotile cells, unable to fertilize the oocyte (Jones, 2002). Produced spermatozoa often carry residual bodies or excess cytoplasm (cytoplasmic droplets) that are phagocytosed by surrounding Sertoli cells. This excess of cytoplasm ensured synchronization of the syncytium of spermatids (Gadella and Luna, 2014), maintaining cross communications/bridges among sister spermatids.

Sperm maturation

The maturation process of spermatozoa is a fascinating journey, intricately influenced by the testosterone. The study of sperm maturation has sparked a growing interest among researchers, leading to a continuous generation of knowledge in this field (Aitken and Baker, 2008; Holt and Morrell, 2013). This process consists of number of changes:

Morphological changes – The excess of cytoplasm in produced spermatozoa is eliminated during the journey within the epididymis. Disruptions in epididymal and/or testicular function can lead to sperm abnormalities, such as cytoplasmic droplets and/or abnormally condensed chromatin. A negative correlation between such abnormalities and fertility has been reported (Sutovsky and Lovercamp, 2010).

Biochemical changes – During epididymal transit, sperm gain motility and fertilizing potential (Sullivan et al., 2005). Biochemical changes alter the sperm plasma membrane's biophysical properties, which is fundamental for the occurrence of subsequent steps providing fertilization capacity to the sperm (Holt and Morrell, 2013). Numerous studies have reported the critical effects of post-gonadal sperm maturation on the sperm cell acquiring complete functionality characteristics (Dacheux and Dacheux, 2014). Epididymal epithelial cells secrete nano- to micro-size vesicles, known as epididymosomes, that contain lipids, proteins, and RNAs (Sullivan et al., 2005; Sharma et al., 2016).

Functional changes – Within the epididymis, the epididymosome vesicles fuse with sperm membranes to induce surface changes and intracellular delivery of their contents, contributing to sperm functionality acquisition (e.g., motility and fertilization ability). Furthermore, during ejaculation, mature epididymal sperm interacts with several molecules contained in secretions from seminal vesicle glands, prostate (prostasomes), bulbourethral glands, and Cowper glands (Rodríguez-Martínez et al., 2011) to further stabilize the sperm membrane by coating their surface (Lu et al., 2011) and protect them during transit through the female genital tract (vagina, cervix, and utero/oviduct) (Gibbons et al., 2005; Troedsson et al., 2005). More specifically, the

prostate gland secretions include the prostasome vesicles, whose lipids, proteins, RNA, and DNA contents also affect sperm function by stimulating motility, regulating capacitation, and protecting against the immune response in the female tract (Aalberts et al., 2014).

Sperm transit within the female reproductive tract

After deposition within the female reproductive tract, spermatozoa that reach the oviduct exhibit good progressive motility, adequate morphology, normal sperm head and size, sensitivity to signaling molecules, and a normal DNA status (Holt and Fazeli, 2015). In the oviduct, epithelial cells and local fluid play a crucial role in modulating sperm function (e.g., capacitation, acrosome reaction, and fertilization) and local gene expression (López-Úbeda et al., 2015).

Sperm capacitation

Capacitation is a process that involves biochemical and physiological modifications on the sperm plasma membrane during the passage of sperm within the female reproductive tract (Gadella and Luna, 2014). This process includes the efflux of cholesterol from the sperm plasma membrane, leading to increased permeability to bicarbonate and calcium ions, and changes in protein phosphorylation. Bicarbonate (HCO_3^-) and calcium ions (Ca^{2+}) play crucial roles in destabilizing the plasma membrane through the cAMP-dependent protein phosphorylation-signaling pathway (Tardif et al., 2001). The activation of soluble adenylyl cyclase generates cyclic adenosine monophosphate (cAMP), in which the calcium ion acts as a secondary messenger, initiating a cascade of protein phosphorylation that triggers sperm motility (Chen et al., 2000; Finkelstein et al., 2020). These controlled alterations activate hyperactive motility and the ability of sperm to undergo an acrosomal reaction when they reach the oocyte (Travis and Kopf, 2002). Overall, the capacitation process could be summarized as successive steps of 1.) acrosome membrane destabilization, 2.) chemical changes in the sperm tail, 3.) increased permeability to Ca^{2+} , and 4.) sperm hyperactivation.

In vivo Capacitation – Sperm capacitation occurs in the oviduct, where sperm cells shed adhering decapacitation factors and interact with ciliated cells of the oviduct epithelium, but mainly with substances present in the oviduct fluids (e.g., glycosaminoglycans). These sperm cells progress towards the ampullary-isthmic junction, where the ovulated oocyte arrests for fertilization (Sostaric et al., 2008; Talevi and Gualtieri, 2010).

In vitro Capacitation – Is induced in a medium containing calcium, bicarbonate ions, and serum albumin, mimicking the ionic and metabolic composition of oviductal fluid (Touré, 2019). Other substances such as cAMP, caffeine, procaine, heparin, progesterone, and methyl β -cyclodextrin are often used for *in vitro* capacitation, a process involving sperm collection from a male donor, their washing in a buffered solution, then incubation in a capacitation medium to initiate capacitation through the synergistic effects of bicarbonate and calcium for boar sperm (Tardif et al., 2001) or negatively charged glycoconjugates for other livestock animals, such as ruminants (Kańska-Książkiewicz et al., 2004). Sperm preparation for *in vitro* fertilization (IVF) consist of their selection (Percoll or swim-up) followed by incubation with matured oocytes in IVF media containing substances that trigger the capacitation process (Henkel and Schill, 2003; Lacalle et al., 2022).

Acrosome reaction and fertilization

The interaction between proteins from the sperm surface and the zona pellucida receptors facilitates the acrosomal reaction. Then, the acrosome-reacted sperm enters the zona pellucida, reaches the perivitelline space, and fuses with the oocyte membrane to transmit the sperm head contents (e.g., DNA, RNA, and protein) into the ooplasm (Coy et al., 2008). Acrosome reaction is controlled by SNARE complexes, leading to the exocytosis of acrosomal contents upon fusion of the plasma membrane with the outer acrosomal membrane. Many studies have used various advanced technologies (e.g., gene silencing, omics) to study critical molecules (e.g., IZUMO1, TMEM95, SOF1, and SPAC6) playing critical roles in sperm-oocyte membrane interaction and fusion (Siu et al., 2021). The functional genome of the spermatozoon, assessed via high-throughput screening methods (omics)

has identified numerous sperm molecules that modulate oxidative phosphorylation, cAMP signaling, and sperm-egg interaction (Johnston et al., 2005; Baker et al., 2007; Hitit and Memili, 2022). As such, biomarkers comprising proteins, mRNAs, lipids, and metabolites hold substantial promise in predicting sperm quality and fertility. These biomarkers have the potential to provide a deep understanding of male reproductive health and sperm phenotype.

Enhancing sperm manipulation through nanotechnology

Screening technologies have revealed specific molecules that are integral to the molecular pathways of sperm biology, influencing various sperm phenotypes (e.g., quality, fertilizing potential, preservation, sex) and male fertility outcomes (Table 1) (Suchocki and Szyda, 2015; Zeng et al., 2021; Dlamini et al., 2023). The unique properties of nanoparticles, such as their small size and high surface area, hold immense potential for their diverse applications in livestock, impacting sperm quality and fertility outcomes (Feugang, 2017; Feugang et al., 2019) (Figure 1).

Table 1. Candidate biomarkers of sperm phenotypes for nanotechnology applications.

Phenotype	Sources	Biomarker candidates	Animals	References
Motility		Proteins: Phosphatidylethanolamine binding protein 4, Spermatogenesis associated 18, Carboxypeptidase, Acrosin	Rams	Zhu et al. (2020)
		Proteins: Mannosidase alpha class 2C member 1, Ubiquinone 1 alpha sucomplex subunit 9-like protein, isoleucyl-tRNA synthetase2, mitochondrial acetyl-CoA acetyl transferase 1, Latherin, Ubiquitin-specific peptidase 43	Horses	Gaitskell-Phillips et al. (2021a)
		Proteins: Hexokinase 1, Aconitase hydratase mitochondrial, Phosphoinositide phospholipase C, Elongation factor Tu, F actin capping protein subunit alpha	Horses	Gaitskell-Phillips et al. (2021b)
		Metabolites: Butyric acid, 1-(2-Methoxy-13-methyl-pentadecanyl)-sn-glycero-3-phosphoethanolamine, 2-O-benzoyl-D-glucose, Trehalulose, Glutamylphenylalanine, Vulgaxanthin-I	Bucks	Yuan et al. (2023a)
		Transcripts: PRM1 mRNAs	Bulls	Ganguly et al. (2013)
Liquid preservation ability/Freezability		Proteins: NUDFB8, SDHC, PDIA4, HSPB1	Bucks	Sun et al. (2023)
		Proteins: GPX5, GLRX, ENO4, QPCT, BBS7, OXR1, DHRS4, AP2S1	Boars	Song et al. (2024)
	Sperm	Metabolites: Oleic acid, Oleamide, N8-acetylspermidine	Boars	Zhang et al. (2023)
		Metabolites: Inosine, Hypoxanthine, Creatine, ADP, Niacinamide, Spermine, 2-methylbutyrylcarnitine	Boars	Torres et al. (2022)
		Metabolites: L-citrulline	Boars	Sui et al. (2023)
		Transcripts: FOS, NFATC3, EAF2, BAMBI, PTPRU, PTPN2, ND6, ACADM,	Boars	Fraser et al. (2020)
		Proteins: Calmodulin, ATP synthase mitochondrial subunits alpha and delta, Malate dehydrogenase and Sperm equatorial segment protein 1	Bulls	Soggiu et al. (2013)
		Proteins: Ras-related protein Rab-2A, Cytochrome b-c1 complex subunit 1, Cytochrome b-c1 complex subunit 2	Boars	Kwon et al. (2015)
	Fertility	Proteins: Acyl-CoA thioesterase 9, Albumin, Casein kinase 2, K voltage-gated channel shaker-related	Bulls	Peddinti et al. (2008)
		Proteins: L amino acid oxidase 1	Bulls	Zhang et al. (2021)
	Metabolites: Gamma-aminobutyric acid, Carbamate, Benzoic acid, Lactic acid, Palmitic acid	Bulls	Menezes et al. (2019)	

Table 1. Continued...

Phenotype	Sources	Biomarker candidates	Animals	References
		Metabolites:	Bulls	DasGupta et al. (2021)
		Taurine, Hypotaurine		
		Transcripts:		
Mitochondrial activity		rRNA genes (18S, 12S, and Large chain R)	Bulls	Lalancette et al. (2008)
		Proteins:		
		Phosphoglycerate mutase, peroxiredoxin 6-like proteins, actin-1 analogue, transmembrane protein analogue		
Viability		Chaperonin TCP1 subunit 8, Testis expressed 101	Stallions	Gaitskell-Phillips et al. (2021b)
Motility		Proteins:	Bucks	Jia et al. (2021)
		Zonadhesin, Superoxide dismutase, Sperm equatorial segment protein 1, Mitochondrial thioredoxin reductase, Zona pellucida binding protein, Aquaporin 7		
		Metabolites:		
		Thioetheramide-PC, Adenosine, N,N-Dimethylguanosine, Isocitric acid		
		Transcripts:		
Seminal plasma		miRNAs (ssc-miR-122-5p, ssc-miR-486, ssc-miR-451, ssc-miR-345-3p, ssc-miR-362, and ssc-miR-500-5p)	Boars	Zhao et al. (2024)
		Metabolites:		
		Propanoic acid, D-ribose, glycine		
Liquid preservation ability/Freezability		Metabolites:	Boars	Song et al. (2023)
		D-proline, Arginine, L-citrulline, Phenylalanine, Leucine, DL-proline, DL-serine, Indole		
		Metabolites:		
		Tryptophan		
Fertility		Ceruloplasmin, Carboxypeptidase E, Serine protease inhibitor family A member 12	Boars	Zeng et al. (2021)

Nanoparticles

Nanoparticles can be naturally or artificially produced in various shapes (such as cubic or spherical) and can consist of inorganic (like metals or salts) as well as organic materials (e.g., lipids, proteins or polymers) (Figures 1 and 2) (Sun et al., 2014). Metal nanoparticles are made in different sizes (ranging from 1 to 100 nm) and shapes (like irregular, rod, spherical, cylindrical, tetragonal, and hexagonal) using inorganic core materials such as cadmium, zinc, gold, silver, platinum, plomb, aluminum, nickel, iron, and copper. The composition of these core materials determines their fundamental properties, like electronical, optical, and physical characteristics, which have garnered much attention in medicine. However, these core materials are often associated with immunogenicity and cytotoxicity of nanoparticles because they can release metallic ions like cadmium (Han et al., 2016; Jain et al., 2018; Kuo et al., 2017; Wang et al., 2020). Nonetheless, coating the core material with multiple layers has helped mitigate its toxicity. For instance, the stabilization of the core material with an inorganic protective shell (e.g., zinc sulfide and silica) and surface modification of the core-shell with various polymers (such as polyvinylpyrrolidone - PVP, polyvinyl alcohol - PVA, polyethylene glycol - PEG, among others), ceramics (like Silicates), and adsorption of anions or charged groups (such as citrate³⁻, chloride ions-Cl⁻, dextran sulfate, polyethyleneimine, etc.) reduces nanoparticle toxicity and enhances biocompatibility (particle dispersion in biofluids), which is essential for bioapplications (Suk et al., 2016; Jain et al., 2018).

Organic nanoparticles (e.g., micelle, dendrimer, liposome, nanogel, and polymeric) contrast their inorganic counterparts. They are formed from aggregated molecules (e.g., polysaccharides and lipids) or polymers of various structures, each exhibiting unique size-dependent physical and chemical properties (e.g., optical and electrochemical). The non-toxic and biodegradable micelles and liposomes, with their hollow spheres, are widely used for pharmaceutical transportation (e.g., drugs and nucleic acids). These organic nanoparticles are designed for bioapplications, and their surface modification with polymers (e.g., PEG) often provides an extended lifespan (Suk et al., 2016; Virlan et al., 2016; Feugang et al., 2022).

Inorganic and organic nanoparticles are biofunctionalized with attached bioactive ligands such as antibodies, oligonucleotides, peptides, and drugs to their external coating layer, providing specificity in cell targeting (Table 2 and Figure 3) (Feugang et al., 2015b; Jain et al., 2018). The high surface area-to-volume ratio and size-dependent physicochemical properties of both inorganic and organic nanoparticles make them versatile and exciting for a wide range of sperm manipulation (Table 2), such as high visibility/contrast in multiple bioimaging, biocompatibility and dispersibility, specific and molecular targeting, and ability to load and deliver diverse cargos for controlled release to target cells (Feugang, 2017; Sutovsky et al., 2024).

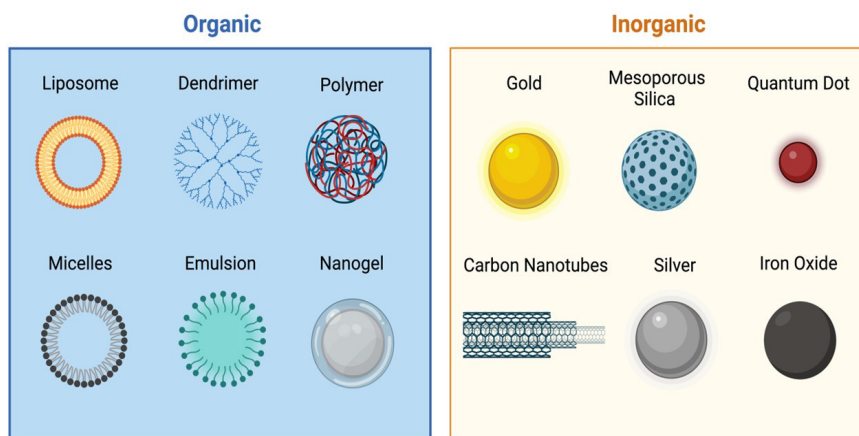


Figure 2. Overview of commonly used inorganic and organic nanoparticles. The presented inorganic nanoparticles can be coated with single or multiple layers of organic or inorganic materials before biofunctionalization for bioapplications. Among the organic nanoparticles, liposomes are the most widely used for bioapplications, such as targeted bioimaging, drug delivery (hydrophobic and hydrophilic), and drug and nucleic acid intracellular delivery. The possibility of coating the external surface of nanoparticles with polyethylene glycol (PEG), or PEGylation, increases nanoparticle's stability and decreases immunogenicity during *in vivo* delivery.

Table 2. Examples of nanoparticles for sperm manipulation.

Classes	Nanoparticles			Biological applications	References
	Types	Shape	Size (nm)		
Organic	Liposomes	Sphere	50-500	Active tumor targeting, drug gene delivery, intracellular delivery, sperm cryopreservation.	Röpke et al. (2011); Deshpande et al. (2013); Mo et al. (2014); Ansari et al. (2016); Feugang et al. (2022)
	Exosomes	Sphere	40 to 120	Sperm function, immune response, cancer therapy, drug delivery	Montecalvo et al. (2012); Du et al. (2016); Fitts et al. (2019)
	Carbon-based: Single-walled (SWCNT)/Multi-walled (MWNT)	Nanotubes	1-200	Gene delivery, sensing, pathogenicity, oxidative stress, inflammation	Donaldson et al. (2006); Donaldson and Tran (2002)
	Polymeric: Nanocapsules/nanospheres (polylactides, polylactide-co-glycolide, chitosan, albumin, gelatin)	Sphere	1-1000	Drug delivery, theragnostics, bioimaging	Szczęch and Szczepanowicz (2020); Zielińska et al. (2020)
Inorganic	Metal nanoparticles: e.g. Gold (Au)	Sphere	2-250	Biomedical imaging, photothermal therapy	Paciotti et al. (2006); Yang et al. (2019)
	Metal oxide nanoparticles: e.g. Fe ₂ O ₃ , CeO ₂	Sphere	15-60	Sperm nanopurification, drug delivery vehicles, thermal-based therapy, sperm bio-imaging	Feugang et al. (2015 ⁹); Falchi et al. (2018a); Arias et al. (2018)
	Non-metal oxide nanoparticles: e.g. Silica (Si)	Sphere	40-100	Purification, gene and drug delivery, biomedical imaging	Wang et al. (2008); Xu et al. (2019)
	Quantum dots	Sphere	1-10	Drug delivery, photodynamic therapy, biomedical imaging, biosensing	Kim et al. (2004); Weng and Ren (2006); Bera et al. (2010); Feugang et al. (2012)

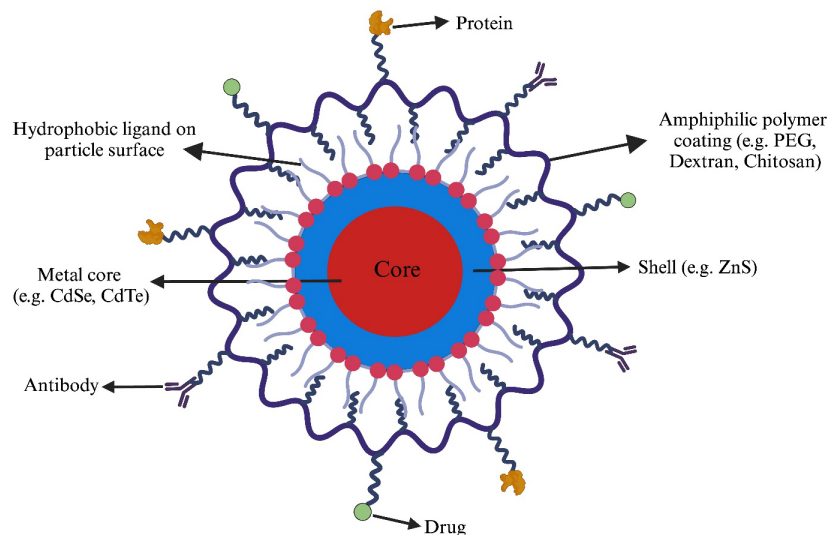


Figure 3. Schematic representation of a biofunctionalized nanoparticle. Ready-to-use nanoparticles for bioapplications include a (nano)core surrounded by single or multiple coating layers providing stability and decreasing immunogenicity and an outer surface layer functionalized based on planned unimodal (e.g., imaging) or multimodal (e.g., imaging and drug administration) applications.

Sperm phenotype and nanotechnology applications

Sperm motility, morphology, and fertility

Sperm motility and morphology are the golden standard for semen evaluation, serving as selection criteria for further processing of ejaculates post-collection. Motility assessment is conducted objectively with automated systems such as computer-assisted sperm analyzer, which allows evaluation of motility and a wide range of kinematic parameters. This assessment, encompassing a diverse range of livestock species, has contributed to establishing a positive correlation between motility parameters and fertility. The research conducted using both fresh or cryopreserved semen (Ansari et al., 2016; Elmi et al., 2018; Lucca et al., 2021; Khan et al., 2024), has consistently shown that high-fertile males tend to exhibit superior total and progressive sperm motility and velocity parameters than their low-fertile counterparts (Vicente-Fiel et al., 2014). These findings suggest that the differential fertility outcomes may be influenced by the morphology and other defects of spermatozoa.

The percentage of morphologically normal spermatozoa is positively correlated with pregnancy rates in all species (Cecere, 2014). On the other hand, sperm defects, such as the aplastic midpiece in bull ejaculates, disrupt sperm functions, especially motility (Díaz-Miranda et al., 2020). Several studies have used proteomics and metabolomics in various livestock species to identify important molecules in sperm associated with morphological defects in the head, midpiece, and tail, which can affect sperm motility and fertility (Peddinti et al., 2008; Menezes et al., 2019; Zhu et al., 2020; DasGupta et al., 2021; Gaitskell-Phillips et al., 2021a; Hitit et al., 2021; Jia et al., 2021; Zeng et al., 2021; Sun et al., 2023). These identified molecules, which may interact with sperm membranes to influence sperm function, can potentially serve as biomarkers for monitoring sperm quality, including motility and morphology. When combining with traditional sperm attributes, these biomarkers may help in selecting the best spermatozoa/ejaculates and predicting fertility more accurately (Sellem et al., 2015; Inanç et al., 2018).

The knowledge of the abovementioned sperm defects with associated biomarkers, such as the lectin and annexin V, targeting damaged acrosome and DNA, respectively, has led to the adoption of different methods of sperm purification using biofunctionalized (or conjugated) nanoparticles to obtain high-quality sperm through targeted elimination of low-quality or immotile sperm in an ejaculate (Feugang, 2017; Meles et al., 2022). Among these techniques, the

density gradient and single-layer centrifugation protocols use nanosized compounds such as the polyvinylpyrrolidone-, silane-, and glycidoxypropyltrimethoxysilane-coated silica colloid solutions, commercialized as Percoll™, Puresperm™, and Androcoll™, respectively. These nanoparticle-based solutions separate pure, high-quality, and viable spermatozoa that have shown numerous applications in reproductive technologies (*in vitro* fertilization, intracytoplasmic sperm injection or ICSI, and artificial insemination) of various livestock species.

Sperm viability

Defects related to membrane (plasma, acrosome, and mitochondrial) losses, capacitation issues, and DNA fragmentation were collectively classified as sperm viability abnormalities (Sutovsky et al., 2024). Sperm membrane integrity is a core attribute of spermatozoa, whose defects are associated with a decrease in the overall functionality of spermatozoa (motility, survival, acrosome reaction, ova binding), leading to early embryonic loss or genetic diseases (Avenidaño et al., 2009; Ferrigno et al., 2021). Plasma membrane defects could be mechanically and/or chemically induced during normal or abnormal physiological processes, leading to damage such as membrane lipid peroxidation and externalization of phosphatidyl serine residues, an indicator of early apoptosis, with subsequent decrease in fertilizing potential (Sutovsky et al., 2024). Acrosome reaction and capacitation are determinant factors of fertilization success that occur during normal physiological conditions, where sperm capacitation is associated with specific markers and molecular pathways related to fertility outcomes (Kwon et al., 2015; Bae et al., 2022). Nevertheless, both acrosome reaction and capacitation processes are affected during semen manipulation (Yoon et al., 2015; Yeste, 2016; Lee et al., 2023). Acrosome-reacted sperm cells exhibit membrane markers that could be targeted for viability evaluation of semen quality. Mitochondrial membrane integrity portrays mitochondria status and energy reserves used by the cell to achieve different physiological functions as a regulator and indicator of sperm motility that could be related to male fertility (Agnihotri et al., 2016; Gallo et al., 2021). Apoptosis and DNA integrity are essential for successfully transmitting the paternal genome, fertilization, and normal embryo development (Kumaresan et al., 2020). Apoptotic spermatozoa are routinely evaluated through Annexin V, which interacts with externalized phosphatidyl serine residues on their plasma membrane. Furthermore, the highly basic protamine 1 protein, participating in DNA packaging, is positively correlated with bull sperm motility (Ganguly et al., 2013) and fertility (Dogan et al., 2015; Llavanera et al., 2021; Souza et al., 2018), with further confirmation in boar spermatozoa (Alvarez-Rodriguez et al., 2021). Interestingly, a recent study demonstrated a positive correlation between DNA fragmentation and protamine deficiency (Kherzi et al., 2019), making protamine a potential target for selecting spermatozoa with high motility and fertility potentials.

The current evaluation methods of semen quality often lead to a binary outcome, categorizing semen as either “Passed” or “Failed” for breeding. Supplementing extenders with organic nanoparticles like loaded or unloaded liposome vesicles and exosomes may reduce the proportion of non-viable sperm cells in semen. Both organic nanoparticles hold promise, demonstrating their potential in repairing damaged spermatozoa through their ability to bind sperm plasma membranes of numerous livestock species (He et al., 2001; Röpke et al., 2011; Pillet et al., 2012; Kumar et al., 2015; Luna-Orozco et al., 2019; Medina-León et al., 2019; Mafolo et al., 2020; Mortazavi et al., 2020). Loaded liposomes and tissular or body-fluids isolated exosomes can be used as nanocarriers for intraspermatic cargo delivery of specific molecules. Unlike the manufactured liposomes that can encapsulate any desired molecules (Feugang et al., 2022) for delivery, exosomes are rich in various molecules (e.g., proteins, miRNA, lipids, metabolites, and mRNA) isolated from various biofluids, including seminal plasma (Piehl et al., 2013; Yang et al., 2017; Dilsiz, 2022; Dlamini et al., 2023). Following binding with spermatozoa, these exosome contents entering spermatozoa may participate in energy pathways, protein metabolism, and maintenance of recipient cells, influencing sperm maturation, capacitation, acrosome reaction, and fertility (Piehl et al., 2013; Du et al., 2016; Qamar et al., 2019). The presence of extracellular vesicles or exosomes in cryopreservation extenders improves post-thaw sperm motility, viability, mitochondrial activity, and membrane integrity (Qamar et al., 2019).

On the other hand, magnetic metal oxide nanoparticles were designed to target damaged cells for removal under a magnetic field to increase the chance of the ejaculated to be approved. In early studies with bovine (Odhiambo et al., 2014) and porcine (Feugang et al., 2015a; Durfey et al., 2019) species, iron oxide nanoparticles were conjugated with lectin, ubiquitin, and annexin V to selectively target and remove acrosome reacted, damaged, and early apoptotic spermatozoa from semen doses without impairing sperm fertility potential.

Sperm freezability

Sperm cryopreservation is a process that allows the long-term storage of semen in liquid nitrogen and is an essential technology for preserving animal fertility (Hungerford et al., 2022). Several sperm functions, including DNA fragmentation, early capacitation, and acrosome and membrane integrity, are impaired during cryopreservation (Yoon et al., 2015; Yeste, 2016), reducing the capacity of spermatozoa to achieve successful fertilization. During this process, spermatozoa experience extreme stress at different proteins, DNA, lipid metabolism levels, and long-chain polyunsaturated fatty acids in the plasma membrane, affecting sperm freezability (Ugur et al., 2019).

Studies of metabolite dynamics in sperm have shown signature differences between fresh, chilled, and frozen-thawed buck (Yuan et al., 2023b) and boar (Torres et al., 2022; Zhang et al., 2023) spermatozoa. Abundance in specific metabolites (e.g., propanoic acid, D-ribose and glycine) in seminal plasma is associated with higher liquid storage ability/freezability of spermatozoa (Pessoa et al., 2023; Song et al., 2023; Zeng et al., 2021). Overall, studies indicate that sperm metabolome changes (e.g., lipids, lipid-like molecules, organic acids and their derivatives) during cryopreservation and influence various biological pathways, such as the linoleic acid metabolism pathway (Yuan et al., 2023b). Further, sperm-borne L-citrulline and seminal plasma-derived tryptophan have been proposed as potential sperm freezability markers in boars (Sui et al., 2023). Similarly, numerous studies have reported the beneficial effects of free amino acids (e.g., alanine, glutamine, histidine, and proline), acting as inhibitors of lipid peroxidation or osmotic regulators. Their presence in extenders or detection in semen has been associated with sperm freezability, thereby enhancing post-thaw viability and quality (Atessahin et al., 2008; Saravia et al., 2009; Trimeche et al., 1999; Ugur et al., 2020).

Similarly, numerous proteomic analyses of sperm ejaculates (sperm and seminal plasma) have unraveled biomarkers (Table 1) and molecular pathways associated with high-quality spermatozoa of many livestock species (Zhu et al., 2020; Gaitskell-Phillips et al., 2021a; Hitit and Memili, 2022). For instance, a study revealed hundreds of differentially expressed proteins between low- and high-freezability buck spermatozoa, with several of these proteins associated with various biological pathways influencing freezability (Sun et al., 2023). Other studies have revealed protein markers (e.g., heat shock protein 90 or HSP90, heat shock protein A8 or HSPA8, or lipocalin-type prostaglandin D synthase or L-PDGS) associated with the sperm freezability status (Ugur et al., 2019). These proteins and metabolites can serve as biomarkers to target high-freezability spermatozoa or could be supplemented associated with nanoparticles in extenders (Table 2). The potential of various nanoparticles, including liposomes and exosomes for successful semen cryopreservation have been evocated in previous works, summarized by (Saadeldin et al., 2020).

Liposome vesicle nanoparticles loaded or not with the identified biomarkers can fuse with the sperm plasma membrane and deliver their contents within the sperm cytoplasm to mitigate sperm damage caused by the freezing-thawing process (He et al., 2001; Purdy and Graham, 2015). The addition of liposomes in egg-free commercial extender (e.g., OptiXcell®) attenuates the damages of bull, buffalo (Ansari et al., 2016), and dromedary camel (Al-Bulushi et al., 2019; Swelum et al., 2019) sperm. Similarly, exosomes with specific contents may play a crucial role in repairing damaged sperm during freezing-thawing (Saadeldin et al., 2020). Furthermore, incubation of spermatozoa with exosomes before cooling or post-thawing increases the antioxidant activity of stored spermatozoa, improving motility, viability, mitochondrial activity, and membrane integrity of post-thaw canine (Qamar et al., 2019) and rat (Mokarizadeh et al., 2013) semen, while decreases the levels of ROS and malondialdehyde

content. Furthermore, classes of nanoparticles can be used to discriminate high versus low freezability spermatozoa for enhanced fertility. The design of magnetic nanoparticle conjugates to target a population of spermatozoa has shown beneficial, with no toxicity effects, in enriching bovine (Odhiambo et al., 2014), equine (Domínguez et al., 2018), and porcine (Durfey et al., 2019) semen with desired sub-populations.

Sperm preservation

Liquid sperm preservation in an appropriate extender is critical for prolonged chilled (15–18°C) or cold (4–10°C) storage, especially in species with poor freezability spermatozoa such as pigs. Harvested semen are generally mixed with extenders containing various protective and nutritive compounds for spermatozoa survival (Kameni et al., 2021; Wiebke et al., 2022), and maintaining extended semen at low temperatures during storage is crucial to reducing sperm metabolism and ATP production, and detrimental byproducts (e.g., reactive oxygen species or ROS) are routine in breeding studs for sperm performance preservation. However, despite these precautions, spermatozoa still experience harmful effects of metabolic decoupling, ion imbalance, activated proteases, cellular acidosis, energy deprivation, and ROS that gradually accumulate in the medium and weaken spermatozoa through multiple damages (Falchi et al., 2018b; Ugur et al., 2019; Kameni et al., 2021).

ROS are among the many toxins the spermatozoa release in the extender, affecting sperm quality during prolonged preservation. The use of antioxidative nanoparticles, such as cerium oxide and vitamin E nanoemulsions, has shown beneficial effects on ram spermatozoa during chilled preservation (Falchi et al., 2018a; Jurado-Campos et al., 2023). Nonetheless, the different abilities of boar semen in sustaining chilling have permitted the identification of differentially expressed proteins (187) of which several were involved in the defense mechanisms against oxidative stress, assembly and maintenance of sperm motility, and sperm metabolism and capacitation. These subsets of proteins could be considered putative biomarkers of sperm quality or preservation (Song et al., 2024). The identified biomarkers are gold mines for the application of appropriate nanoparticles to discriminate high versus low sperm preservability. There is potential in designing magnetic nanoparticle conjugates to selectively target a population of spermatozoa, thus enriching semen doses with high freezability spermatozoa. Furthermore, the application of magnetic nanoparticle conjugates to target the identified protein marker candidates has potential to contribute to enhanced fertility outcomes of preserved semen (Odhiambo et al., 2014; Feugang et al., 2015a).

Semen redox status

Oxidative stress results from the excess of ROS produced during the oxygen metabolism of spermatozoa. To maintain optimal sperm function, a balance between oxidation and reduction is necessary, involving the neutralization of ROS through antioxidants. The antioxidative status has been linked to sperm fertility potential, having beneficial roles at normal levels during acrosome reaction and fusion with the oocyte. Rupture of the redox equilibrium leads to oxidative stress, lipid peroxidation, and subsequent alterations in sperm characteristics and fertility (Gundogan et al., 2010; Sapanidou et al., 2023). Redox disequilibrium is aggravated during post-collection semen manipulation (centrifugation, freezing, thawing, and incubation) causing damage to sperm membranes rich in polyunsaturated fatty acids. Lipid peroxidation has shown a negative correlation with sperm characteristics of bulls (Castiglioni et al., 2021), while high-fertility bulls spermatozoa showed less susceptibility to lipid peroxidation and overexpressed transcripts associated with the reduction process compared to their low-fertile counterparts (Alvarez-Rodriguez et al., 2021; Saraf et al., 2021; Leite et al., 2022). Meanwhile, the oxidative stress index of chilled-stored boar semen showed a negative correlation with sperm motility and *in vivo* fertility (Barranco et al., 2021). The mitigation of the adverse effects of oxidative stress during preservation includes supplementation of semen extenders with various antioxidants (Amidi et al., 2016; Hosseinmardi et al., 2022; Kameni et al., 2022), providing extended protection to spermatozoa during storage. Innovative approaches for

sustainable protection of spermatozoa involve utilizing nanoparticles, which show potential as antioxidants owing to their inherent redox activity. These nanoparticles aid in neutralizing and alleviating oxidative stress within extenders during (cryo)preservation (Asadi et al., 2023). Hence, the intrinsic antioxidative effects of several metal oxides (e.g., cerium, cupric, and zinc oxides), vitamin E nanoemulsions, and noble metals (e.g., gold and platinum) have been shown to offer beneficial effects, such as improved post-storage sperm motility and membrane integrity (Falchi, et al., 2018c; Valgimigli et al., 2018; Hashem and Gonzales-Bulnes, 2020; Hosseinmardi et al., 2022; Jurado-Campos et al., 2023; Khaliq et al., 2024) These antioxidant nanoparticles in (cryo) preservation media improve the spermatozoa's redox status and sperm attributes, which may positively impact pregnancy rates.

Furthermore, unilamellar liposome vesicles, prepared exosomes, and mesoporous nanoparticles can be loaded with different antioxidants for controlled release during storage. Liposomes loaded with antioxidants (e.g. quercetin, lycopene) and other lipid types (e.g., soybean lecithin) provide greater cryoprotection of spermatozoa of livestock species such as bovine (Röpke et al., 2011; Kumar et al., 2015), porcine (He et al., 2001), ovine (Luna-Orozco et al., 2019; Mafolo et al., 2020; Mortazavi et al., 2020), and equine (Pillet et al., 2012; Medina-León et al., 2019).

Sperm sexing

Sperm sexing is a powerful tool that selectively separates X and Y chromosome-bearing sperm cells to produce animals of predetermined sex (Yata, 2021). Flow cytometry is the modern conventional method for sperm sexing that uses lasers to differentiate between sperm genders based on their DNA content (Ugur et al., 2019). Sperm sexing offers advantages, and the commercial demand for sexed semen is increasing in cattle. However, the resulting low yield of sexed sperm numbers becomes a significant challenge, with species like pigs and horses requiring more sperm per insemination dose (Quelhas et al., 2023). A recent proteomics study has identified specific proteins of the bull sperm membrane, of which 12 and 3 were upregulated in X- and Y-bearing spermatozoa, respectively (Quelhas et al., 2021). These proteins could be potential candidates for immunoselection using nanotechnology tools like magnetic nanoparticles. A large-scale production of magnetic nanoparticles is needed, and their surface modification to target differential negative charges between X and Y sperm has successfully targeted Y sperm, leaving semen enriched with X spermatozoa (Dominguez et al., 2018).

Conclusions and perspectives

Advanced technologies have enabled a deeper understanding of sperm biology and the processes involved in sperm phenotype and physiological status. Identifying biomarkers linked to sperm phenotype and their combination with specific nanoparticles offer new opportunities for precision breeding in livestock. This technological approach can be extended to sperm manipulation to enhance the success of various sperm biotechnologies, such as (cryo)preservation, sexing, and sperm-mediated gene transfer. Further research using nanotechnology tools is essential to improve the success rate of assisted reproduction in livestock, especially during critical seasons and instances of animal diseases.

List of abbreviations

ACADM: Acyl-Coenzyme A dehydrogenase medium chain
ADAM2: Disintegrin and metalloproteinase domain-containing protein 2
AI: Artificial insemination
AP2S1: Adaptor related protein complex 2 subunit sigma 1
ATP: Adenosine triphosphate
BAMBI: BMP and activin membrane bound inhibitor
BBS7: Bardet-Biedl syndrome 7
cAMP: Cyclic adenosine monophosphate
DHRS4: Dehydrogenase/reductase 4
DNA: Deoxyribonucleic acid
EAF2: ELL associated factor 2

ENO4: Enolase 4
FOS: Fos proto-oncogene
GLRX: Glutaredoxin
GPX5: Glutathione peroxidase 5
HSP90: Heat shock protein 90
HSPA8: Heat shock protein A8
HSPB1: Heat shock protein family B (small) member 1
IZUMO1: Izumo sperm-egg fusion protein 1
L-PDGS: Lipocalin-type prostaglandin D synthase
miRNA: Micro ribonucleic acid
mRNA: Messenger ribonucleic acid
MWNT: Multi-walled carbon nanotube
ND6: Nicotinamide adenine dinucleotide + hydrogen dehydrogenase subunit 6
NFATC3: Nuclear factor of activated T cells 3
NUDFB8: Nicotinamide adenine dinucleotide + hydrogen: Ubiquinone Oxidoreductase Subunit B8
OXR1: Oxidation resistance 1
PDIA4: Protein disulfide isomerase family A member 4
PE: Polyethylene
PEG: Polyethylene glycol
PTPN2: Protein tyrosine phosphatase non-receptor type 2
PTPRU: Protein tyrosine phosphatase receptor type U
QPCT: Glutaminy-peptide cyclotransferase
RNA: Ribonucleic acid
ROS: Reactive oxygen species
rRNA: Ribosomal ribonucleic acid
SDHC: Succinate dehydrogenase complex subunit C
SNARE: Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SOF1: Sperm-oocyte fusion required 1
SPAC6: Sperm acrosome associated 6
SWCNT: Single-walled carbon nanotube
TMEM95: Transmembrane protein 95

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Author contributions

SLK: Methodology, Writing-Editing; NHD: Methodology, Writing-Editing; JMF: Conceptualization, Supervision, Writing, Reviewing, Funding acquisition. All authors read and approved the manuscript.