Micro- and Nanoscale Approaches in Antifungal Drug Discovery

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Abstract: Clinical needs for novel antifungal agents have increased due to the increase of people with a compromised immune system, the appearance of resistant fungi, and infections by unusual yeasts. The search for new molecular targets for antifungals has generated considerable research, especially using modern omics methods (genomics, genome-wide collections of mutants, and proteomics) and bioinformatics approaches. Recently, micro- and nanoscale approaches have been introduced in antifungal drug discovery. Microfluidic platforms have been developed, since they have a number of advantages compared to traditional multiwell-plate screening, such as low reagent consumption, the manipulation of a large number of cells simultaneously and independently, and ease of integrating numerous analytical standard operations and large-scale integration. Automated high-throughput antifungal drug screening is achievable by massive parallel processing. Various microfluidic antimicrobial susceptibility testing (AST) methods have been developed, since they can provide the result in a short time-frame, which is necessary for personalized medicine in the clinic. New nanosensors, based on detecting the nanomotions of cells, have been developed to further decrease the time to test antifungal susceptibility to a few minutes. Finally, nanoparticles (especially, silver nanoparticles) that demonstrated antifungal activity are reviewed.

Keywords: antifungal drug discovery; microfluidics; nanobiotechnology; omics-based approaches; antifungal susceptibility testing; nanomotion detection; nanoparticles

1. Introduction

Fungal infections are an extremely important health problem. Fungi infect about 1.2 billion people every year, yet their contribution to the global burden of disease is largely unrecognised [1,2]. Over 600 different fungi have been reported to infect humans. Most are "relatively" minor infections, but millions contract diseases that kill at least as many people as tuberculosis or malaria. More than 300 million people are affected by serious fungal infections worldwide. Invasive fungal infections are responsible for about 1.5 million deaths per year. Fungi are present everywhere in our environment and are, usually, harmless for people with a normal immune system. Fungal infections can be topical and local, such as infections on the skin or in the vaginal tract. Systemic infections arise when the fungi enter and proliferate in the bloodstream. Systemic fungal infections affect people with an altered immune system due to medical interventions (such as cancer therapy, organ transplantation, and immune-modulatory medications), immunosuppressive diseases (such as Acquired Immune Deficiency Syndrome (AIDS)) [3], or malnutrition (under- and overnutrition) [4–6].

The choice of available antifungal drugs to treat invasive fungal infections is limited, since only three structural classes of compounds are available, i.e., polyenes, azoles, and echinocandins. Antifungal therapy has become progressively more effective since the 1990s, however, no new

antifungal classes have been reported since 2006 [7]. Current antifungal drugs show some limitations: Amphothericin B (a polyene antibiotic) displays a considerable toxicity and undesirable side effects [8,9], issues with pharmacokinetic properties (such as a short half-life of echinocandins) and activity spectrum, a small number of targets [10,11], and they can interact with other drugs, such as chemotherapy agents and immunosuppressants [12,13]. The last approved antifungal (i.e., anidulafungin) by the European Medicines Agency and the Food and Drug Administration (FDA) dates back to 2006 [14]. There is an urgent need for safer and more effective antifungal drugs. Multiple types of antifungal compounds are in clinical development and these new agents have been recently reviewed [15–17].

Over the last 20 years, several approaches to antifungal discovery have been explored. The traditional approach seeks, first, to identify active compounds from large compound libraries using a panel of fungal pathogens in standardised assays when possible. In the genetic, genomic, or bioinformatics approach, the objective is, initially, to identify broadly represented targets in fungal pathogens and non-pathogens [18]. In this "target-centric" genomic approach, up-front genetic, bioinformatics, and biochemical target prioritisation is performed and, subsequently, an in vitro-based screening of individual targets is achieved. However, an exceedingly high rate of failure has been observed when applying this approach [19]. Additionally, not all bioactive compounds act through a target-specific mechanism of action (MoA). Various compounds act non-selectively as alkylating agents, intercalators, detergents, etc. Therefore, an integrated approach has been recently proposed for antimicrobial lead discovery that is rooted in empirical whole-cell screening for small molecules with intrinsic bioactivity whose MoA may be determined using a variety of forward or reverse genomic platforms to identify and, subsequently, validate their target [20]. In this "compound-centric" strategy, it is proposed to use existing genetic methods to evaluate the therapeutic effect of inactivating the target (both in vitro and in vivo). These methods often use the principles of genetic interaction, relying on the idea of genetic modifiers (enhancers or suppressors) to generate target hypotheses. Direct biochemical methods or computational inference have also been used for target identification. In many cases, however, a combination of approaches may be required to fully characterise on-target and off-target effects, and understand the mechanisms of small-molecule actions [21].

Recently, micro- and nanoscale approaches have been introduced in the field of antifungal drug discovery. In this paper, microfluidic approaches for antifungal screening and antifungal susceptibility testing (AFST), nanosensor development for AFST, and antifungal nanoparticles are reviewed.

2. Emerging Fungal Diseases and Antifungal Drugs

The epidemiology of invasive fungal infection is evolving [22–25]. A growing population of immunosuppressed patients have been diagnosed with invasive fungal infections. The epidemiology of candida infections has shifted over the last decade [26]. Non-*albicans Candida* species, with a reduced susceptibility to antifungals, are becoming more and more responsible for invasive candidiasis [27]. Several species, such as *Candida glabrata* and *C. krusei*, which are less susceptible to azole antifungal therapy, are increasing in some settings. *C. auris* has emerged in hospitals as a global concern, since the strains demonstrated multidrug resistance. *Trichosporon* species infections are the second most common cause of fungaemia in patients with haematological malignant disease and they were the third most commonly isolated non-*candida* species from patients in the ARTEMIS Global Antifungal Surveillance Program [28]. These species show resistance to amphotericin and echinocandins. *Rhodotorula* species are emerging opportunistic yeasts that are responsible for catheter-related fungaemia and sepsis, and invasive infections, particularly in immunosuppressed or -compromised patients [24]. The incidence of infections with non-*neoformans* crytococci has increased over the past 40 years, especially in patients with advanced HIV infection or cancer who are undergoing transplant [29]. Since the 1990s, there have been a growing number of reports about *Saccharomyces cerevisiae* invasive infections and novel strains continue to be identified [24,30,31].

Aspergillus species have, historically, been one of the most common causative organisms associated with invasive mould diseases [32]. *Aspergillus fumigatus* species were responsible for most of infections. Recently, the epidemiology has changed towards non-*A. fumigatus* species and other moulds, such as *Zygomycetes* [33–36], *Fusarium* [37,38], and *Scedosporium* [38,39] species. This is due to the occurrence of azole resistant *Aspergillus* species [40,41].

Antifungal development is challenging, since, apart from the fungal cell wall, fungi are metabolically similar to mammalian cells and, therefore, offer few pathogen-specific targets [42]. As explained above, only three molecular classes that target three different fungal metabolic pathways are commonly used in clinical practice to treat, essentially, systemic fungal infections: Polyenes, azoles, and echinocandins (Table 1) [43]. The fluoropyrimidine analog 5-fluorocystosine is also used, but only in combination with amphotericin B.

Table 1. Overview of the mostly used antifungal agents, mechanism of action (MoA), and spectrum of activity [43–50].

Class Compound	MoA	Spectrum of Activity	Comment
Polyenes			
Amphotericin B	Selective binding to ergosterol cause the formation of pores in the membrane.	Treatment of deep mycoses, candidiasis, cryptococcosis, histoplasmosis, blastomycosis, paracoccodioidomycosis, coccidioidomycosis, aspergillosis, extracutaneous sporotrichichosis, and some cases of mucormycosis, hyalohyphomycosis, and phaeohyphomycosis, *S. cerevisiae*.	Fungicidal, broad spectrum, intravenous, little resistance observed, significant nephrotoxicity, indirectly affects action of many drugs.
Nystatin/Nyotran	Selective binding to ergosterol cause the formation of pores in the membrane.	Candidiasis	Nyotran is a liposomal formulation of nystatin with lowered toxicity.
Natamycin	Binds to ergosterol in the plasma membrane, preventing ergosterol-dependent fusion of vacuoles, membrane fusion, and fission.	Keratinophilic fungi, corneal infections	
Azoles			
Fluconazole	Selective inhibition of fungal cytochrome P450-dependent lanosterol-14-α-demethylase.	*Candida immitis*, *C. neoformans*, *Paracoccidioides brasiliensis*; lower activity against *Aspergillus*, *Fusarium*, *Scedosporium*, *Penicillium* species and other filamentous fungi.	Fluconazole resistant *C. albicans* and non-*albicans* strains increasing.
Itraconazole	Selective inhibition of fungal cytochrome P450-dependent lanosterol-14-α-demethylase.	Most *Candida* species, *P. brasiliensis*, *H. capsulatum*, *Blastomyces dermatitidis*, *Aspergillus fumigatus*, *A. niger*, *Penicillium marneffei*.	Better than fluconazole in the treatment of coccidioido-mycosis, not reaching the central nervous system; numerous drug interactions due to inhibition of CYP 3A4.
Voriconazole	Selective inhibition of fungal cytochrome P450-dependent lanosterol-14-α-demethylase.	*Candida* species, including *C. krusei*, *S. cerevisiae*.	More potent than fluconazole; very rapid metabolism in children; numerous drug interactions due to inhibition of CYP 3A4.
Econazole	Selective inhibition of fungal cytochrome P450-dependent lanosterol-14-α-demethylase.	*Trichophyton rubrum*, *T. Mentagrophytes*, *Epidermophyton floccosum*.	Is an immidazole antifungal for the treatment of tinea pedis and crusis, pityriasis versicolor.
Tioconazole	Selective inhibition of fungal cytochrome P450-dependent lanosterol-14-α-demethylase.	*C. albicans*, *Trichophyton* sp., *Epidermophyton* sp.	Is an immidazole antifungal for topical treatment of superficial mycoses (ringworm, jock itch, athlete's foot, tinea versicolor.

Table 1. *Cont.*

Class Compound	MoA	Spectrum of Activity	Comment
Echinocandins			
Caspofungin	Fungal β-1,3-glucan synthase inhibitors.	*Candida* species, *Aspergillus* species.	Fungicidal for *Candida*, fungistatic for *Aspergillus;* modest efficacy as first-line agent for invasive aspergillosis; intravenous formulation only; interacts with ciclosporin and rifampicin.
Anidulafungin	Fungal β-1,3-glucan synthase inhibitors.	*Candida* species, *Aspergillus* species.	Fungicidal for *Candida*, fungistatic for *Aspergillus*; licensed for the treatment of invasive and esophageal candidiasis.
Micafungin	Fungal β-1,3-glucan synthase inhibitors.	*Candida* species, *Aspergillus* species.	Fungicidal for *Candida*, fungistatic for *Aspergillus*; licensed invasive and esophageal candidiasis.
5-Fluoropyrimidine			
5-Fluorocystosine	Selective conversion of toxic intermediate (5-fluorouridine).	Cryptococcosis, candidiasis, chromoblastomycosis; high MIC for some strains of *Aspergillus*, *Penicillium* and several Zygomycetes, except for chromoblastomycosis.	5-Fluorocystosine is always used in combination with amphotericin B.

Recently, a lot of effort has also been made in analysing the antifungal action of natural compounds or natural bioactive compounds. The discovered natural antifungal derivatives or compounds derived from natural origins have been recently reviewed [17,51]. Some examples of these compounds include chitosan [52]; herbal compounds, such as thymol, carvacrol, eugenol, and menthol [53]; and extracts from plants, such as *Hypericum carinatum* [54], *Stenachaenium megapotamicum* [55], and *Acca sellowiana* [56].

3. Omics-Based Antifungal Drug Discovery Approaches

The traditional approach seeks, first, to identify active compounds (generally from large libraries of synthetic small molecules or natural products) that inhibit the growth of the fungus. The most commonly used assay to identify antifungal leads is liquid growth inhibition assay in which microbial growth is measured by optical density (OD) of the culture. These assays have some limitations, such as, sometimes, a poor correlation between growth and OD for fungi that grow as filaments, and these assays are unable to distinguish between molecules that inhibit growth (fungistatic) from those that kill the organism (fungicidal), a feature that is very important for the treatment of some fungi (e.g., *Cryptococcus*) [57]. Recently, cell viability screening assays based on other readouts have been developed. The most widely adopted approach is the use of the dyes, Alamar Blue and tetrazolium salt (XTT), as reporters for metabolic activity. These dyes are converted to fluorescent molecules when metabolised by viable molecules and can be used for high-throughput multiwell screening [58,59]. The dye, resazurin, was used to develop an assay for high-throughput screening of *A. fumigatus* [60]. An Alamar-Blue-based high-throughput protocol was devised to identify molecules with fungicidal activity against *C. neoformans* [61]. A second type of viability assay that was recently developed is based on the detection of extracellular adenylate kinase (AK) as a reporter of cell lysis [62]. AK is a conserved enzyme that has been used as a cytotoxicity reporter in mammalian cell culture assays and as a reporter of brewing yeast autolysis in the beverage industry [63].

Another approach is referred to as genetic, genomic, or bioinformatics in which the objective is, initially, to identify broadly represented targets in fungal pathogens and non-pathogens. The operational code for this strategy consists of three basic rules for identifying potential targets: (i) A bioinformatics-driven approach is used to identify pathogen-specific genes with the desired conservation and spectrum, and the absence of a human ortholog; (ii) genetic analyses are performed to confirm that loss-of-function mutations result in a nonviable growth phenotype and/or a nonvirulent

phenotype under standard laboratory conditions and/or a nonvirulent phenotype in a relevant animal model of infection; and (iii) there should be some evidence, derived from sequence, structure, or biochemical information that the target is druggable [64]. This operational code was used to guide genomic and target-based antimicrobial discovery. Forward and reverse chemical-genetic methods have been used to identify the target of drug-like bioactive compounds and MoA. Target-based approaches have been pursued using high-throughput screening for inhibitor of enzymes and/or proteins that perform essential functions in microbes. Molecular target-based screening has provided mediocre to poor results in the field of anti-infective drug discovery, suggesting that cell-based screening for antimicrobials remains the most effective strategy [19]. Gene knockout microorganisms and small molecules with well-defined mechanisms can each be used to alter the functions of putative targets, uncovering dependencies on activity. Reverse chemical screens that exploit genome-wide collections of mutants have been employed to map susceptibility phenotypes to specific genes by systematically screening antimicrobial agents against a defined (ideally) comprehensive mutant collection [20]. Mating of laboratory and wild yeast strains can reveal patterns of small-molecule sensitivity with specific loci [65]. In another method, molecularly barcoded libraries of open reading frames have been used to detect small-molecule-resistant clones that are then identified by microarray analysis [66].

A proteomic profiling approach, based on two-dimensional difference gel electrophoresis and mass spectrometric identification of the proteins, has been used to predict the target protein of small molecules of interest [67]. It was demonstrated that this proteomic profiling system could discriminate small molecules by their mechanism of action. Omics methods have also been used successfully to identify the MoA of compounds that were discovered using traditional screening approaches. A key illustrative case is the discovery of the orotomide F901318 compound [68]. A combination of genetic and biochemical approaches revealed the target of F901318 in *A. fumigatus*, which was initially discovered in a high-throughput screen of compound libraries. It was found that F901318 acts via the inhibition of the pyrimidine biosynthesis enzyme, dihydroorotate dehydrogenase, in a fungal-specific manner.

Although only around 1100 of the 6000 genes of *S. cerevisiae* are essential under nutrient-rich growth conditions [69], almost all genes become essential in specific genetic backgrounds in which another non-essential gene has been deleted or otherwise attenuated, an effect termed synthetic lethality [70]. Genome-scale surveys suggest that over 200,000 binary synthetic lethal gene combinations dominate the yeast genetic landscape [71]. The genetic buffering phenomenon is also manifested as a phalanx of differential chemical-genetic interactions in the presence of sublethal doses of bioactive compounds [72]. These observations highlight the inherent redundancy of genetic networks and frame the problem of interdicting network functions with single agent therapeutics [73]. This genetic network organisation suggests that judicious combinations of small molecule inhibitors of both essential and non-essential targets may elicit additive or synergistic effects on cell growth [74,75]. Compounds that enhance the activity of known agents in yeasts have been identified by small molecule library screens [76–79]. Direct tests of synergistic compounds have successfully yielded combinations that are active against pathogenic fungi, such as the combination of fluconazole with chemical inhibitors (such as 17-AAG and 17-DMAG) of Hsp90 [80], calcineurin (such as miconazole, ketoconazole), or adenosine diphosphate (ADP) ribosylation factor (ARF) (such as brefeldin A [81], the antibiotic polymyxin B [78]), and compounds selected from an off-patent drugs library [79]. Combinatorial antifungal therapies have many advantages, including a decrease in the rate of selection of resistant strains, a lower required dosage of individual drugs, a decrease in host toxicity, and enhanced antifungal activity [82]. Syncretic combinations of drugs with improved antifungal properties can be readily identified in both model fungal species and highly pathogenic clinical isolates [83]. Additionally, it was shown that synergistic combinations usually yield enhanced selectivity without adverse side effects [84].

These, recently developed, omics-based approaches require expensive, automated, robotic screening platforms when applied in high throughput. Additionally, these platforms are usually

based on multiwell screening, which has some limitations. As discussed in the next section, further improvements can be achieved by integrating these methods in microfluidic screening platforms.

4. Micro- and Nanoscale Approaches

4.1. Microfluidic High-Throughput Antifungal Drug Discovery

Growing cells in 96-, 384-, or 1536-well plates has miniaturised cell assays for drug discovery. These experiments with multiwell plates are typically integrated in a robotic analysis platform. Major drawbacks of robotic platforms are the expense of the instrumentation, the cost of experimental consumables, the systems are closed (no flow through of reagents or cell culture medium), and still a relatively high consumption of reagents compared to recent developed microfluidic chips. Additionally, as cell collections are growing, there is a need to further miniaturise the assays to increase the parallelism of analyses.

In microfluidic lab-on-a-chips, fluids are manipulated at the micrometer length scale [85]. Reducing the scale evidently reduces the reagent consumption and, consequently, the cost to perform assays, which becomes significant for high-throughput drug screening. The physics at micro/nano scale are exploited in microfluidic chip designs for drug discovery. Other physical phenomena dominate at this small length scale compared to the macroscale [86]. Fluid flow in microchannels is characterized by a low Reynolds number (a dimensional criterion that determines the relative importance of inertial and viscous effects). The dominant role of viscous forces results in laminar flow behavior and mass transport by diffusion between two adjacent fluids, resulting in the generation of stable concentration gradients [87]. Also, fast media and temperature changes can be obtained using the laminar flow [88]. Mass transport by diffusion becomes a fast mixing method when the length scale is reduced in small channels or wells. It has been demonstrated that, at these small length scales, time-resolved reactions have been conducted with millisecond resolution due to the extremely rapid diffusive mixing [89,90]. Surface tension plays an important role in the formation of small droplet emulsions in immiscible fluids in microfluidic channels [86]. Surface tension and viscous stresses destabilize the interface and create droplets when water is injected into a stream of oil at a T-junction or two perpendicular crossing microchannels [91]. Based on this principle, droplet-based microfluidic platforms have been developed to encapsulate and screen single cells [92].

Due to the ease of controlling the cellular environment, microfluidic technology has been used to perturb cellular physiology in screening arrays. Additionally, the cell number and density of a given area or volume can be controlled, which allows monitoring of a high spatial and temporal resolution and observation of the dynamic behavior of many cells [93,94]. Parallellisation of experimental conditions and automatization in microfluidic chips has resulted in designs for high-throughput cell screening [95], such as living cell microarrays [96–101]. Microfluidic parallelization and single-cell monitoring allows the measurement and averaging of parameters on hundreds of individual cells, compared to measuring parameters of a whole cell population. New insights can be obtained by observing single cells, such as the monitoring of certain classes of proteins, using fluorescently tagged proteins [102,103] and give information about the cell-to-cell variation in a heterogeneous microbial population [104–106]. Automation of microfluidic cell culture systems also leads to standardized manipulation and monitoring, which allows the perfect timing of protocols to characterize dynamic processes at high temporal resolution to be performed [94].

Cellular microarray platforms have been developed for high-throughput antifungal drug discovery based on the screening of nanoliter biofilms that are created in hydrogels (Table 2). Robotic printing is used to fabricate the cellular arrays. The formation of biofilms complicates antifungal therapy, since the ability of fungal cells to form biofilms is an important reason for the emergence of severe resistance to most clinically available antifungal agents [107–110]. A better understanding of fungal biofilms provides new opportunities for the development of urgently needed novel antifungal agents and strategies.

Table 2. Examples of microfluidic platforms for high-throughput antifungal drug discovery.

High-Throughput Technology	Application	Microorganism	Characteristics	References
Cellular microarray	Antifungal biofilm screening	*Candida albicans*	Cells robotically printed, 768 (48 × 16 array) cultures of 50 nL biofilms in collagen.	[111,112]
Cellular microarray	Antifungal biofilm screening	*C. albicans*	Cells robotically printed, 1200 (60 × 20 array) cultures of 30 nL biofilms in alginate.	[113]
Cellular microarray	Antibiotic and antifungal biofilm screening	*C. albicans*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*	Robotically printing of mono- and polymicrobial biofilms, 576 (48 × 12 array) cultures of 30 nL biofilms in alginate.	[114]
Droplet microfluidics	Antifungal drug screening	*Phytophthora sojae*	The plant pathogen spores and the drug were encapsulated in liquid droplets. Phenotypic responses to the drug at different concentrations were microscopically quantified.	[115]

4.2. Microfluidics for Antifungal Susceptibility Testing

Resistant microbial infections are becoming a major threat to public health, causing increasing mortality worldwide. The fast emergence of multiresistant pathogens is caused by extensive, and sometimes unnecessary, use of antimicrobials and the lack of interest in developing new variants. The cost of antimicrobial resistance (AMR) is projected to increase significantly as some models predict a rise in global casualties from the present figure of one million to 10 million in 2050 [116]. A survey of antimicrobial susceptibility test (AST) methods available demonstrated a need for new approaches that would enable rapid, inexpensive, and sensitive tests that can quickly provide physicians with antibiotic profiles [117]. A quick diagnostic method is necessary to prescribe a patient that is infected by a life-threatening microbe with an effective antimicrobial [118]. AST is widely applied to determine antimicrobial resistance profiles of the microbial isolates and to help in the selection of an antimicrobial treatment option [117,119–121]. Currently, AST is usually not performed in the clinic, but in a clinical microbiology lab, which necessitates transportation of the patient samples [122]. Current susceptibility tests, that are based on cell culturing of the pathogen, can take several days (especially for slow growing microbes) and extends the time to make the correct diagnosis and decisions for appropriate and effective antimicrobial therapy. This leads to increased patient mortality and the use of broad-spectrum antimicrobials that promote resistance [120]. To survive this evolutionary war against microbial pathogens, we must pursue technologies that can rapidly perform AST to enable personalized therapies (narrow-spectrum antimicrobial administration) at the earliest possible treatment stage [122].

Standard antifungal susceptibility (AFST) methods rely on measuring fungal growth in the presence of antifungals over a few days. The broth microdilution (BMD) is the standard method for the evaluation of susceptibility to antifungal agents in *Candida* species [123–126]. Standardized micro-dilution-based procedures by the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antibiotic Susceptibility Testing (EUCAST) are universally accepted for performing AFST [127]. However, these procedures are complex, time-consuming, and not intended for routine use.

Commercially available tests, such as Sensititre YeastOne (broth microdilution method), Etest (agar-based disk diffusion method), and the fully automated Vitek 2 (broth microdilution method) yeast susceptibility system, all easy-to-use modifications from CLSI/EUCAST reference methods, are widely used for testing antifungal susceptibility of relevant *Candida* and *Aspergillus* species [10]. New diagnostic approaches based on emerging technologies, such as flow cytometry, MALDI-TOF mass spectroscopy, and isothermal microcalorimetry, have been developed to expand, and potentially improve, the capability of the clinical microbiology laboratory to yield AFST results [126]. Flow cytometry is used to determine the effect of an antifungal compound by measuring changes in the

viability of fungal cells that are fluorescently labeled [128,129]. A simple and rapid AFST assay based on MALDI-TOF was developed [130]. This approach facilitated the discrimination of the susceptible and resistant isolates of *C. albicans* after a 3-h incubation in the presence of "breakpoint" level drug concentrations of the echinocandin caspofungin (CSF). A microcalorimetry-based AFST assay has been developed for real-time susceptibility testing of *Aspergillus* spp. [131]. The method is based on measuring changes in growth-related heat production in the presence of the antifungal compound.

Semi-automated susceptibility systems (such as VITEK and MicroScan) decrease the turn-around-time and operator touch-time (culture and colony isolation are still required) compared to traditional culture-based methods, but can add significant cost to the tests [132]. The fastest test still requires 9 h and, therefore, these semi-automated systems do not provide information in time to influence initial treatment decisions [133,134].

Since microfluidics promises several advantages over existing macro-scale methods, several microfluidic platforms that can perform rapid antimicrobial susceptibility tests have been developed during the last years (Table 3) [132,135]. The recent development of microfluidic platforms was mostly focused on devices for antibiotics susceptibility testing and much less on antifungals susceptibility testing, although the same designs could, usually, be used for both (e.g., see [136,137]). Most systems rely on microscopic transmission or fluorescence observation of cells to quantify the effect of the antimicrobial on the cell growth or viability. The microorganisms are confined to a small volume (wells, channels, chambers, or droplets). In many systems, the cells are immobilized in a hydrogel, such as agarose or alginate (Table 3) (Figure 1).

Table 3. Examples of microfluidic platforms for antimicrobial susceptibility testing.

Microorganism	Measurement Principle	Description	Reference
Fungi			
Candida strains	Fluorescence-based distinction between living and dead cells.	Cell Chip kit [1] used as cell sorter and the determination of fluorescence histograms of previously labelled cells.	[138]
Candida albicans	Immunosorbent ATP-bioluminescence assay.	The microfluidic device employs a fiberglass membrane sandwiched between two polypropylene components, with capture antibodies immobilised on the membrane. Cells immobilised in alginate hydrogel.	[136]
Saccharomyces cerevisiae	Fluorescence staining and imaging after incubation.	Cell seeding and diffusive medium supply is provided by phase-guide technology, enabling operation of continuous culturing.	[137]
Bacteria			
Escherichia coli	Optical imaging of single cell growth (number of cells).	Growth of cells in channels, with a large surface-to-volume ratio.	[139]
E. coli	Magnetic bead rotation, which is inversely proportional to bacterial mass.	Droplet microfluidics where single cells are entrapped in liquid drops.	[140]
E. coli	Optical imaging of single cell growth (number of cells).	Individual cells grow in gas permeable (PDMS) microchannels, with dimensions comparable to a single cell.	[141]
E. coli	Fluorescence imaging of cells that express green fluorescent protein.	Growth of cells in 12 sets of quadruplicate microfluidic chambers. Quantification of the effect of four antibiotics and their combinations.	[142]
E. coli	Reflectometric interference spectroscopy of pH-sensitive chitosan hydrogel measured the accumulation of metabolic products.	Growth of cells in microfluidic channels.	[143]
E. coli	Fluorescence imaging of immunomicrobeads attached to the cells.	Growth in microfluidic chambers.	[144]

Table 3. *Cont.*

| Microorganism	Measurement Principle	Description	Reference
Bacteria			
E. coli	Optical imaging.	The standard broth microdilution method was miniaturised in a microfluidic chip that generates an antibiotic concentration gradient and delivers antibiotic-containing culture media to eight 30-nL chambers for cell culture.	[145]
E. coli	Fluorescence imaging.	The microfluidic chip allows the carrying out of commonly executed antibiotic susceptibility assays in an array of nanoliter droplets.	[146]
E. coli	Optical phase-shift reflectometric interference spectroscopic measurements.	The use of biofunctionalised silicon micropillar arrays to provide both a preferable solid-liquid interface for bacteria networking and a simultaneous transducing element that monitors the response of bacteria when exposed to chosen antibiotics in real time.	[147]
E. coli	Spectral absorbance of cell suspensions.	An automated linear gradient generator based on centrifugal microfluidics.	[148]
E. coli, Nitrosomas europaea	Optical imaging of single cell growth (number of cells).	The cells grow in a layer of agarose upon which a gradient of the antibiotic is applied.	[149]
Enterobacter cloacae, E. coli, Klebsiella pneumoniae, P. aeruginosa, Acinetobacter baumannii	Optical imaging of bacterial replication.	A solid-phase microwell growth surface in a 384-well plate format was used, with inkjet printing–based application of both antimicrobials and bacteria at any desired concentrations.	[150]
Enterococcus faecalis, E. coli	Fluorescence staining and imaging after incubation.	Cell seeding and diffusive medium supply is provided by phase-guide technology, enabling operation of continuous culturing.	[137]
Mycobacterium tuberculosis	Optical imaging of single cell growth (number of cells).	Cells were immobilized in an agarose matrix, which was molded in a microfluidic chip.	[151]
Pseudomonas aeruginosa	Fluorescence imaging of GFP-expressing cells.	The microfluidic chip generates a logarithmic concentration gradient through semidirect dilution in a zero-flow condition and cells grow in nanoliter reactors.	[135]
Staphylococcus aureus	Fluorescence imaging of viability indicator.	Stochastic confinement of individual cells into liquid plugs (droplet microfluidics); distinction between sensitive and resistant bacteria.	[152]
S. aureus	Optical imaging of single cell growth (number of cells).	Antibiotics diffuse into a microfluidic channel containing the cells.	[153]
S. aureus	Fluorescence imaging for dead cells (rates of killing).	Cells are covalently bound to the bottom of the channels and fluid flow shear stress activation of pathways that are targets of antibiotics.	[154]
S. aureus	Optical imaging of single cell growth (number of cells).	Chip with 32 individual fluidic channels.	[155]
S. aureus, E. coli, K. pneumoniae, P. aeroginosa	Optical imaging of single cell growth (number of cells).	Antibiotics diffuse into a microfluidic agarose channels in 96-well format.	[156]
S. aureus, S. epidermitis, S. saprophyticus, E. coli, K. pneumonia, P. aeruginosa, P. mirabilis, Streptococcus pyrogenes, S. viridans	Immunosorbent ATP-bioluminescence assay.	The microfluidic device employs a fiberglass membrane sandwiched between two polypropylene components, with capture antibodies immobilised on the membrane. Cells immobilised in alginate hydrogel.	[136]
Salmonella thyphimurium, E. coli, S. aureus	Optical imaging of single cell growth (number of cells).	Cells immobilised in agarose with a gradient of the antibiotic in the gel slab.	[157]
P. aeruginosa	Optical imaging of single cell growth (number of cells).	Growth of cells in four parallel channels.	[158]

[1] Agilent Technologies.
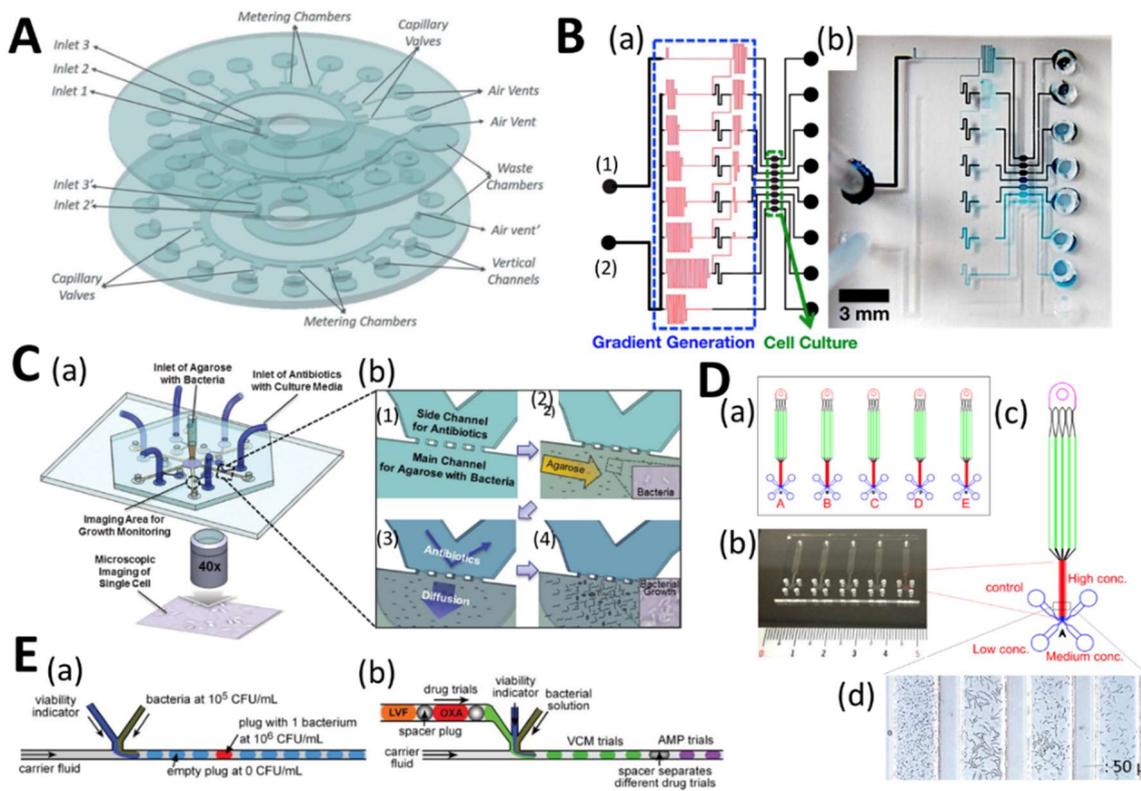
Figure 1. (**A**) An automated linear concentration gradient generator based on centrifugal microfluidics for antibiotic susceptibility testing (AST). The operation is based on the use of multi-layered microfluidics in which individual fluidic samples to be mixed together are stored and metered in their respective layers before, finally, being transferred to a mixing chamber. Reprinted with permission from [148]; (**B**) (**a**) The microfluidic microchip design: Black and red channels represent 149-µm and 4-µm tall features, respectively; (**b**) A photograph of the fabricated PDMS chip. Blue food dye solution and water were injected to upper (1) and lower (2) inlet ports. (**a**) Reprinted with permission from [145]; (**C**) Schematic diagram of the AST process for the microfluidic agarose channel (MAC) system. (**a**) The MAC chip was fabricated with PDMS on glass. An agarose–bacteria mixture solution was injected into the center of the chip, which flowed synchronously into the six main channels. Each interface between the agarose with the bacteria and antibiotic solutions was monitored microscopically to analyse bacterial cell growth. (**b**) (1) The empty channel before AST. (2) The bacteria were mixed with agarose and then injected into the main channels. (3) A sharp interface was generated due to the anchors and then liquid medium with different concentrations of antibiotic. (4) Time-lapse microscopic observation of a bacterial cell. Reprinted with permission from [153]; (**D**) The structure of the AST microfluidic device for MIC determination of five drugs. (**a**) Design. (**b**) Actual image. (**c**) Precise structure of one set of fluids. (**d**) Microscopic image of *Pseudomonas aeruginosa* grown in the presence of piperacillin. Reprinted with permission from [158]; (**E**) Stochastic confinement of bacteria into plugs. (**a**) Schematic drawing illustrates the increase in cell density, resulting from the stochastic confinement of an individual bacterium in a nanoliter-sized plug. (**b**) Screening of many antibiotics against the same bacterial sample. Antibiotics: Ampicillin (AMP), levofloxacin (LVF), vancomycin (VCN), and oxacillin (OXA). Reprinted with permission from [152].

4.3. Nanomotion Analysis for Evaluating Antifungal Suscessibility

Recently, very new sensitive sensor technologies based on microcantilevers have been developed [159–161]. Nanomechanical oscillators are being used for the detection of very small masses [162,163], for measuring buoyant mass, and determining the "instantaneous" growth rates of individual cells [164] for the quantitative time-resolved membrane protein (vancomycin)—ligand (mucopeptides) on cantilever arrays with 10 nM sensitivity and at clinically relevant concentrations in blood serum [165]. Cantilever nanosensors have been used to measure mass differences in the pico- to femtogram ranges in air [166] and liquid [167,168]. Many of the available systems are limited by the need to perform the measurements in air or in a humid environment [161].

Microcantilevers have been explored as nanosensors for living cell studies, since they offer many advantages, such as being highly sensitive, selective, label-free, performed in real time, and provide in situ detection capabilities [169]. Single cell detection and monitoring on the cantilever sensor has been reported for *S. cerevisiae* cells [164,170,171], *E. coli* [164,171,172], *Bacillus subtilis* [164,172], *Enterococcus faecalis* [171], HeLa cells [173], mouse lymphoblasts [164], and human lung carcinoma and mouse lymphocytic leukemia cells [171,174], and mouse and human T cells [171]. Cell growth detection has been demonstrated by monitoring resonance frequency changes of cantilevers as the mass increases from immobilized *S. cerevisiae* and fungal *A. niger* spores on the surface of the cantilevers in humid air [175]. *S. cerevisiae* cells were deposited onto the cantilever surface and its bending as a function of time and corresponded to the yeast growth behaviour [169]. Recently, serial microfluidic mass resonator arrays were used to measure single-cell growth of yeast, bacterial and mammalian cells in liquid and a higher throughput [171].

Recently, a nanomechanical detector, that can be used to assess the effects of chemicals on living organisms in a timeframe of minutes, was developed. In this technique, the living microorganisms are adsorbed to the surface of a nanomechanical sensor, i.e., an AFM cantilever, and its fluctuations are measured as a function of time (Figure 2). This approach requires only a few minutes without cell growth, since the decrease in cell activity in the presence of the antimicrobial is measured. Moreover, it is a label-free technique. These are major advantages compared to current methods. Antimicrobials can be introduced in the measuring chamber, allowing a rapid identification of the antimicrobial to which the microorganism is susceptible. The sensor position detection is similar to the one used in Atomic Force Microscopy (AFM): A laser beam is focused onto the sensor (cantilever) and ends its path onto a multisegment photodiode. Numerous proof-of-concept experiments have been performed involving several species of bacteria and fungi. By comparing this method with the traditional techniques in double-blind experiments, a success rate close to 90% could be achieved [176]. The variation of the nanovibration signal is directly proportional to the metabolic activity of the microbial cell. It allowed estimating with unpreceded speed (15 min per ampicillin dose), the minimum inhibitory (MIC) and bactericidal concentrations (MBC) for the effect of the antibiotic ampicillin on *E. coli* [177]. The high sensitivity of the nanosensor allowed differentiation between bacteriostatic (kanamycin) and bactericidal (ampicillin) effects when exposed to a resistant *E. coli*. The high sensitivity of the nanosensor made it possible to differentiate between bacteriostatic and bactericidal effects. Recently, this method was successfully applied to blood culture pellets to determine the antibiotic susceptibility against agents of bloodstream infection [178]. AFM-nanomotion detection (NMD) has also been applied for AFST, where the effect of a low (10 µg/mL) and high (40 µg/mL) caspofungin concentration on *C. albicans* was evaluated [179]. It was recently demonstrated that AFM-cantilever NMD is so sensitive that single-cell activities of *S. cerevisiae* cells could be detected [179], and even cell cycle progression could be observed [180].
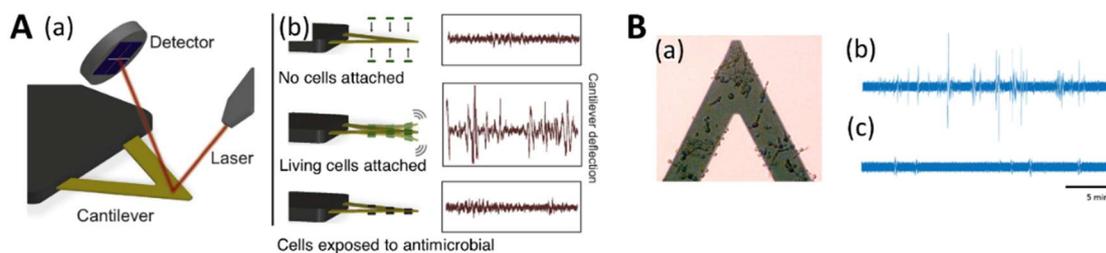
Figure 2. (**A**) Outline of experimental setup and description of experiments. (**a**) Schematics of nanomotion detector setup with a cantilever sensor; laser beam is focused on the surface of the sensor and reflection is used to monitor movements of the cantilever. (**b**) Representation of a typical nanomotion susceptibility test. When microorganisms are not attached to the sensor, fluctuations are driven only by thermal motion and are relatively low. After attachment of living cells, fluctuations are linked to their metabolic activity and are high. Finally, after exposure to an antimicrobial drug, the cells are nonviable and fluctuations return to low levels. Reprinted with permission from [178]. (**B**) (**a**) *C. albicans* deposited onto a cantilever. (**b**) Reprinted with permission from [161].

4.4. Antifungal Nanoparticles

Due to the development of antimicrobial resistance, pharmaceutical companies and researchers are searching for new antimicrobial agents. Nanoscale materials have emerged as novel antimicrobial agents, owing to their high surface area to volume ratio and their unique chemical and physical properties [181,182]. By definition, nanoparticles are structures that have dimensions in the 1–100 nm [183]. Silver nanoparticles (Ag-NPs) are one of the most commonly used nanomaterials in consumer and medical products because of their antimicrobial activity [184,185]. It is increasingly used in a variety of both medical and consumer products, resulting in an increase in human exposure [183,186]. A large number of in vitro studies indicate that Ag-NPs are also toxic to mammalian cells derived from the skin, liver, lung, brain, vascular system, reproductive organs, and the immune system. Although significant progress has been achieved on the elucidation of the antimicrobial mechanism of silver nanoparticles, the exact mechanism of action is still not completely known [187]. It has been demonstrated that non-cytotoxic doses of Ag-NPs could induce genes that are associated with cell cycle progression [188,189] and apoptosis in human hepatoma cells [189]. DNA damage by Ag-NPs in mammalian cells has also been reported [188,190]. The mechanisms for Ag-NP induced toxicity include the effects of this particle on cell membranes, mitochondria, and genetic material. It has been recently demonstrated that bacteria (*E. coli*, *P. aeruginosa*) can also develop resistance to silver nanoparticles after repeated exposure [191]. The biosynthesis of silver nanoparticles is now considered to be the most environmentally friendly and cost-effective method [185]. It can be achieved using a variety of organisms, such as by the fungi, *A. flavus* [185], *A. terreus* [192], *Arthroderma fulvum* [193], *Penicillium fellutanum* [194], and *P. expansum* [192].

Other nanostructures have been discovered that also show antifungal activity. It was demonstrated that cationic terephthalamide-bisurea molecules showed excellent microbial selectivity, with minimal host toxicity [195]. The terephthalamide-bisurea recognition motif facilitated spontaneous supramolecular self-assembly, with the formation of fibres in water. Antifungal activity against clinically isolated, drug-sensitive, and drug-resistant *Cryptococcus neoformans* strains was observed. These antifungal agents showed effectively dispersed *C. albicans* biofilms and excellent in vivo biocompatibility. Homogeneously dispersed copper nanoparticles (originating from n-Cu sepiolite fibres) in soda-lime glass powder showed high antibacterial properties against gram-positive (*Micrococcus luteus*) and gram-negative bacteria (*E. coli*), and antifungal (*Issatchenkia orientalis*) activity [196]. The observed high activity of the n-Cu glass powder was explained by the inhibitory synergistic effect of the Ca^{2+} lixiviated from the glass on the growth of the cells, since n-Cu sepiolite had no significant antifungal activity. Another approach of obtaining nanoparticles with antifungal activity is to immobilize, covalently, the antifungal amphotericin into nanomaterials, such as silica

nanoparticles [197]. These antifungal nanoparticle conjugates demonstrated fungicidal activity against several strains of *Candida* sp., mainly by contact. In addition, they could be reused for up to five cycles without losing their activity. The results showed that the antifungal nanoparticle conjugates were more fungistatic and fungicidal than 10 nm colloidal silver.

5. Conclusions

Fungal infections continue to appear as the population of people with an altered immune system increases. An impaired immune system arises due to medical intervention, immunosuppressive diseases, or malnutrition. The infections become life-threatening for systemic infections. The therapeutic options for invasive fungal infections are limited, since a very limited number of structural classes of drug compounds are available and some demonstrate significant limitations. The discovery of new antifungals is mostly achieved by the screening of natural or synthetic/semisynthetic chemical compounds [16,17]. Using the genomics approach, substantial progress has been made in antifungal drug development for a multitude of potential drug targets [198] and inhibitors [199,200]. Recent advances that could support and refine the antifungal pipeline were focused on elucidating fungal pathways, targets, and mechanisms of action that could lead to new antifungal therapies; antifungal compounds and immune strategies currently in development that could become new antifungal therapies; improved formulations of existing compounds; and the repurposing of drugs approved for other indications and could show potential antifungal activity [16]

Surely, there is a need for novel antifungal discovery approaches. The current antifungal tools that are available to tackle the invasive fungal epidemic occurring in clinics and hospitals have improved, but are still inadequate for use in all patient groups [16]. Recently, micro- and nanoscale approaches have been introduced in antifungal drug discovery. It has become increasingly clear in trends in antimicrobial drug discovery that microfluidic approaches will have an increasing role. The capability of manipulating fluids, flexibility on geometries and materials, manipulation of a large number of cells simultaneously and independently, and ease of integrating numerous analytical standard operations and large-scale integration makes microfluidic devices a versatile tool for antifungal drugs. One of the future challenges lies in the construction of extended cell microarrays, or single-cell droplet arrays, and integration in the microfluidic chip [100], since the recently developed bioinformatics approach for antifungal discovery could be further improved by integrating these methods in a microfluidic screening platform. Living cell arraying methods, based on closed microchambers arrays in microfluidic bioreactors, could increase throughput significantly compared to classical multiwell-plate cell assays, with significantly reduced amounts spent on expensive test reagents, cells, and chemical compounds, and without the need for expensive robotic multiwell-plate screening facilities.

Significant progress has been made in the development of microfluidic platforms for antimicrobial susceptibility testing (AST). Recent efforts are focused on the development of AFST methods that are independent of cell growth and can provide susceptibility results in a very short time frame. These methods rely on measuring the cell death in the presence of the antifungal. A promising technique is the AFM-based nanomotion detection, which can give an answer about the susceptibility in a few minutes. Further parallelization of this method is required to introduce it in the clinic.

Nanoscale materials have emerged as novel antimicrobial agents. One of the most extensively studied nanoparticles are silver nanoparticles and this is for their antifungal, but also antibacterial and antiviral, activities. In minute concentrations, it was found not to be toxic to humans and microorganisms [185]. Further research should focus on a better understanding the toxicity and determining the exact mechanism of the interaction between nanoparticles and cells.

NanoMicrobiology (NAMI), and the International Joint Research Group (IJRG) VUB-EPFL BioNanotechnology & NanoMedicine (NANO).

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