REVIEW

Current status and applications of aminoglycoside microarrays

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Abstract: Amino glycosides, one of the important broad-spectrum antibacterials, treat clinically against various bacterial infections. In the last decade, amino glycoside micro arrays have become one of main technologies for analyzing interactions between antibiotics and therapeutic targets in a high-throughput manner. A series of methods have been developed to immobilize amino glycosides on the functional group-coated glass slides in a micro array format. The amino glycoside micro arrays technology has been widely used for rapid determination of interactions of the amino glycosides with ribosome RNAs (rRNA) and proteins. Several clinically used amino glycosides are mainly exerted by binding to bacterial rRNA, which leads to mistranslation of protein. However, amino glycosides are losing efficacy due to the increased resistance mostly caused by enzymes modification. The micro array-based technology is mainly used in developing novel antibiotics, discovering new RNA targets, and identifying inhibitors of resistance-causing enzymes. This review will focus on the construction of amino glycoside micro arrays, their recent status and applications in biological and biomedical research and some challenges in further research.

Keywords: Amino glycoside micro arrays, rRNA, resistance, acetyl transferase, resistance-causing enzymes, RNA-binding molecule.

INTRODUCTION

Generally, the term "aminoglycosides (or aminoglycoside antibiotics)" refers to a group of antibiotics, which contain a molecular nucleus, two or more amino sugars linked to the nucleus with glycosidic bonds and an aminocyclitol ring of streptidine or 2-deoxystreptamine (Kondo *et al.*, 1999). There are several useful typical aminoglycosides including a newest amino glycoside (ACHN-490) in clinical trials as shown in fig. 1. Amino glycosides are broad-spectrum antibiotics against both Gram-positive and Gram-negative bacterial infections, especially the anaerobic Gram-negative infections. In 1944, streptomycin, the first amino glycoside, was extracted from the metabolins of *Streptomyces griseus*, for the treatment of bacterial infections, such as tuberculosis (Schatz *et al.*, 1944). Streptothricin B (fradiomycin) (Umezawa et al., 1948) and neomycin (Waksman *et al.*, 1949) were later discovered and applied to clinical use. However, the emergence of resistant bacteria of antibiotics limited their continued intensive clinical use. Umezawa and co-workers (1957) discovered kanamycin (produced by *Streptomyces kanamyceticus*) for the treatment of drug resistant bacteria, such as streptomycin-resistant tuberculosis. However, kanamycin also resulted in the resistant strains in patients in a low incidence with widely used in the world. The biochemical mechanisms of resistance to aminoglycosides were then studied (Umezawa *et al.*, 1967; Bryan *et al.*, 1988; Benveniste *et al.*, 1973; Doi *et al.*, 2007; Xiao *et al.*, 2012) and the enzymatic mechanisms were first illustrated in 1967. It was demonstrated that three types of aminoglycoside resistance-causing enzymes are involved in the enzymatic mechanisms, which are aminoglycoside acetyltransferases (AACs), amino glycoside nucleotidyltransferases (ANTs) and amino glycoside phosphoryltransferases (APHs) (Llano-Sotelo *et al.*, 2002).

Then many aminoglycosides have been discovered or semi-synthesized, such as amikacin, sisomicin, netilmicin and isepamicin (Waitz *et al.*, 1972; Kabins *et al.*, 1976; Minguez *et al.*, 1989). However, no aminoglycoside has been launched for a long time since the semi-synthetic amino glycoside arbekacin was approved in 1990. The newest semi-synthetic aminoglycoside ACHN-490 (fig. 1), a derivative of sisomicin, was discovered in 2009 (Endimiani *et al.*, 2009) and applied in phase III clinical trials now. It has been indicated that ACHN-490 could target bacterial ribosome with high binding affinity and be resistant to most of aminoglycoside resistance-causing enzymes (Aggen *et al.*, 2010; Armstrong *et al.*, 2010; Labby *et al.*, 2013).

The binding between amino glycosides and the aminoacyl-tRNA site (16S rRNA A-site) in bacterial ribosomes could lead to the inaccurate translation of bacterial protein and then cell death (Magnet et al., 2005). Modification effect of resistance-causing enzymes on

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aminoglycosides can decrease their binding affinity for therapeutic targets and then cause resistance (Walsh *et al.*, 2000). In order to fight with the increased incidence of resistant bacteria, novel aminoglycosides should have the ability to bind strongly and specifically to therapeutic targets and not bind or bind weakly to resistance- and toxicity-causing enzymes. However, traditional aminoglycoside profiling methods, such as mass spectrometry, fluorescence spectrophotometry and surface plasmon resonance, were highly material-consumed (Ding *et al.*, 2003; Shandrick *et al.*, 2004; Verhelst *et al.*, 2004). Recently, microarray technique has been developed as one of high-throughput methods to identify novel antibiotics (Ratner *et al.*, 2004).

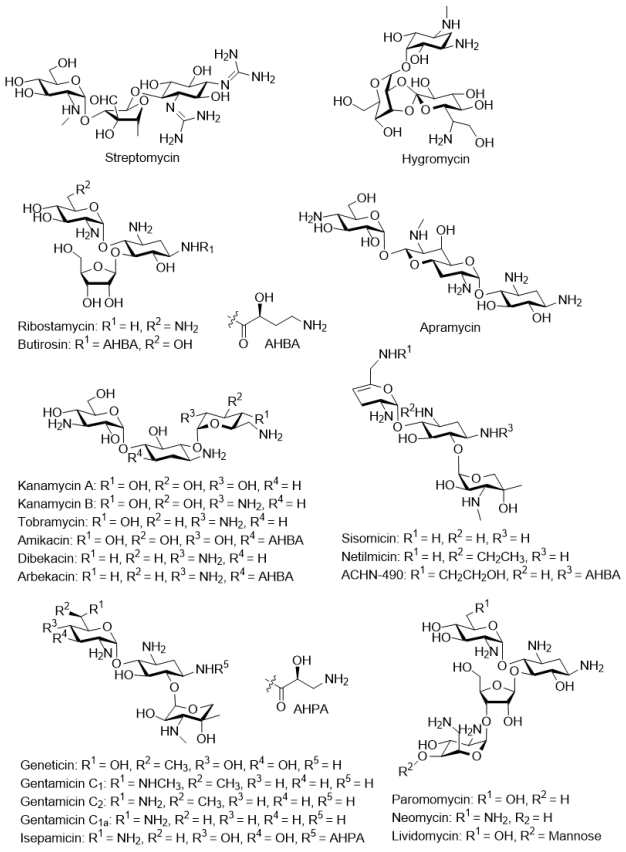

Fig. 1: Structures of clinically useful typical aminoglycosides including the newest aminoglycoside (ACHN-490) in clinical trials

Carbohydrate microarrays, which construct hundreds to thousands of different glycan moieties onto solid surfaces in a spatially discrete model, are universal and sensitive methods to study carbohydrate-protein interactions (Fukui *et al.*, 2002; Disney *et al.*, 2004; Horlacher *et al.*, 2008; Geissner *et al.*, 2014). In 2004, Seeberger's group (Disney *et al.*, 2004) firstly used the aminoglycoside microarray to research interactions of antibiotics with RNAs and proteins, which aroused many other scientists' interests in this field (Guan *et al.*, 2012; Barrett *et al.*, 2008; Disney *et al.*, 2008; Tran *et al.*, 2010). Our review gives a quick overview of the fabrication, recent status and applications of aminoglycoside micro arrays.

Fabrication of aminoglycoside microarrays

The fabrication methods of aminoglycoside microarrays are similar to other carbohydrate microarrays. Construction of renewable and reliable methods to immobilize aminoglycosides on the solid surfaces is the key element in the whole fabrication of aminoglycoside microarrays. So far, non-covalent and covalent immobilization methods have been developed, which can be further classified into site-nonspecific and site-specific attachment (Park *et al.*, 2013). Non-covalent immobilization of aminoglycosides is achieved by the adsorption of aminoglycosides onto solid surfaces. Although non-covalent immobilization is easy to perform, it has several drawbacks, for example aminoglycoside is immobilized to the slide surfaces by nonspecific attachment, and aminoglycoside is too small to provide a large enough contact area to interact efficiently on the solid surfaces. Covalent immobilization of aminoglycosides immobilizes aminoglycosides to derivative solid surfaces through covalent bonds. Up to now, the site-specific and covalent immobilization is the most widely used fabrication method of the aminoglycoside microarrays and will be introduced in this review.

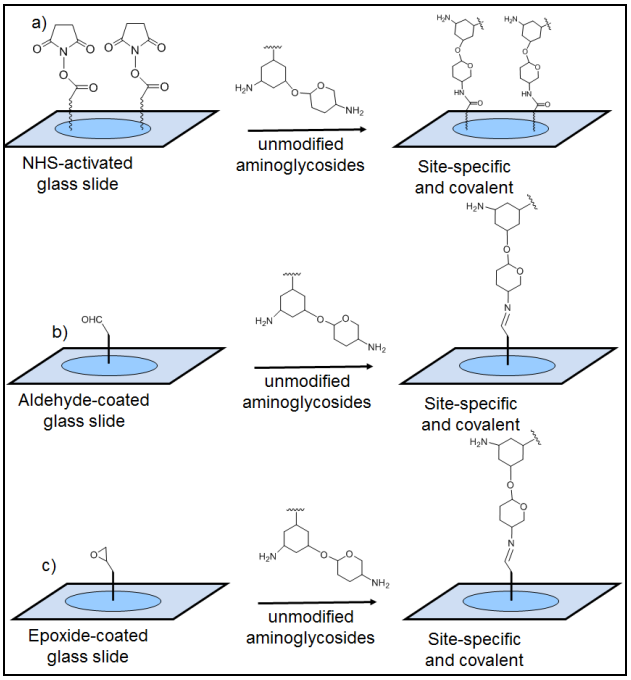

Fig. 2: Covalent, site-specific immobilization of unmodified aminoglycosides on (a) glass slides coated with *NHS* ester; (b) aldehyed-coated glass slides; and (c) epoxide-coated glass slides

Immobilizations of unmodified aminoglycosides on derivatized surfaces

Since modification of aminoglycosides is quite time-consuming and labor-demanding, unmodified

aminoglycosides can be simply covalently immobilized to the derivative surfaces by one-step procedure. As shown in fig. 2, Seeberger's group (Disney *et al.*, 2004) developed a procedure in which unmodified aminoglycosides were covalently immobilized to the functional group-coated glass slides. They used *N*-hydroxysuccinimide (*NHS*)-activated glass slides for immobilization of aminoglycosides through amido bond (fig. 2a). There are two ways to prepare the *NHS*-activated glass slides. One is to treat amine-coated glass slides with tetraethyleneglycol disuccinimidyl disuccinate and the other one is to treat amine-coated glass slides with *N,N'*-disuccinimidyl carbonate and bovine serum albumin. Meanwhile, they also used the aldehyde-coated glass slides for immobilization of aminoglycosides by carbon-nitrogen double bond (fig. 2b).

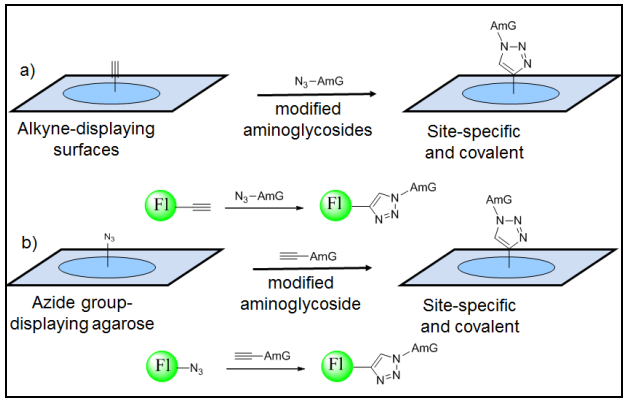

Fig. 3: Site-specific immobilization of chemically modified aminoglycosides on derivative surfaces (a) immobilization of azido-aminoglycosides onto alkyne-functionalized agarose or conjugation to fluorescein (Fl); (b) immobilization of alkyne-aminoglycosides onto azido-functionalized agarose or conjugation to fluorescein (Fl); AmG refers to aminoglycoside

In our study, we found that amino glycosides can also be used to immobilize with epoxide-coated glass slides (fig. 2c) (unpublished data). Other functional group-coated glass slides are urgently required for the efficient immobilization of unmodified amino glycosides.

Immobilizations of chemically modified amino glycosides on derivatized surfaces

Aminoglycoside microarrays can also be constructed in a covalent manner to attach chemically modified aminoglycosides to derivative surfaces. In this approach, proper functional groups bound to aminoglycosides can facilitate the identification of novel aminoglycosides with specific and strong binding to their desired targets. Thus, the selection of suitable functional groups is one of the most important steps of the structural modification of aminoglycosides. Disney MD and his colleagues (2008) modified aminoglycosides with azido group and then constructed microarrays on the alkyne-displaying agarose or conjugation with fluorescein (Fl) by a Huisgen dipolar cycloaddition reaction (fig. 3a). Using the same reaction, they also modified aminoglycosides with the alkyne group and constructed microarrays on the azido-displaying surfaces (fig. 3b) (Disney *et al.*, 2007).

Recent applications of aminoglycoside microarrays

Since the carbohydrate microarray technology was developed in 2002, the scope of applications of aminoglycoside microarrays has been rapidly expanded. Many problems can be solved through high-sensitivity and high-throughput aminoglycoside microarrays. The aminoglycoside micro arrays can be mainly applied to develop novel antibiotics with strong affinity to RNA targets but weak affinity to resistance- and toxicity-causing enzymes by determining their interactions. Moreover, the aminoglycoside microarrays have potential application in the discovery of inhibitors of resistance-causing enzymes, which may be used by co-administration with antibiotics to reduce the risk of drug resistance.

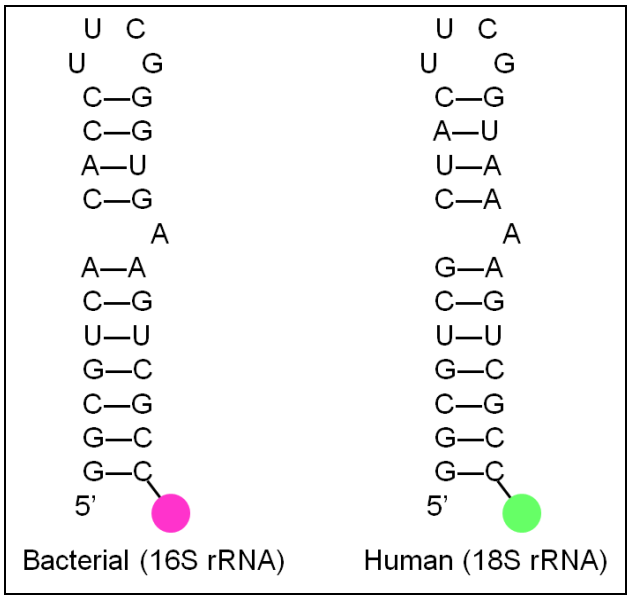

Fig. 4: The oligonucleotide mimics of rRNA A-site mimics of prokaryotic and eukaryotic ribosome, which only differs at a single base pair (the bacterial A1408 corresponds to G1408 in human), were incubated with the aminoglycoside arrays. Each oligonucleotide was fluorescently labeled. The bacterial RNA is labeled with tetramethyl rhodamine (TAMRA) and the human with fluorescein

Applications in antibiotics development

Interactions with RNA targets

It is well known that the bactericidal activity of aminoglycosides is mainly upon the inhibition of the synthesis of protein through binding to the 16S rRNA A-site in bacterial ribosomes. Therefore, RNAs are potential targets due to their involvement in various crucial biological processes, such as mRNA splicing, protein synthesis, transcriptional regulation and retroviral

replication (Johnstone *et al.*, 2001; Bayne *et al.*, 2005; Fedor *et al.*, 2005). There is a tremendous interest in developing small molecules that can specifically and effectively bind with RNA.

To search the potential antibiotics targeting RNA, Seeberger's group (Disney *et al.*, 2004) constructed aminoglycoside micro arrays. The antibiotics were covalently immobilized onto *NHS*-reactive glass slides in a random way by an automated arraying robot. This method provides a flexible platform for identifying the interactions of aminoglycosides with different targets, including RNAs and proteins. Arrays were probed with two different species of RNA sequences, a bacterial 16S rRNA mimic and a human 18S rRNA A-site mimic (fig. 4) (Disney *et al.*, 2004). The binding affinities of a range of aminoglycosides and some other small molecules to rRNAs were determined and compared. It showed that these aminoglycosides have different binding abilities to the rRNA (16S or 18S) and each aminoglycoside recognizes different rRNA mimics with different affinities (the binding affinity for 16S rRNA mimic is at least one order of magnitude stronger than that for 18S rRNA mimic). Among these aminoglycosides, amikacin binds to both the human and the bacterial A-site mimics with the highest affinity.

Fig. 5: An array of guanidinoglycosides and aminoglycosides, which were incubated with fluorescein-labeled AAC (6'). Arrows point to 3×3 blocks of repetitive spots (spot diameter ≈150 μm) on the array that correspond to 1: β-Ala-neomycin, 2: β-Ala-guanidinoneomycin, 3: β-Ala-ribostamycin, and 4: β-Ala-guanidinoribostamycin

Aminoglycoside microarrays could not only bind with short RNA oligonucleotide mimics of a large RNA, but also bind directly with large RNA. A group I intron ribozyme with about 400 nucleotides in length, which is the potential binding target for many aminoglycosides inhibiting group I intron self-splicing, was chosen to test with aminoglycoside microarrays (von Ahsen *et al.*, 1993; Disney *et al.*, 2001; Adams *et al.*, 2004; Stahley *et al.*, 2005). The results showed that the interactions of large RNAs with arrayed aminoglycoside could be detected, among which amikacin displayed the highest binding affinity (Disney *et al.*, 2004).

Some scientists (Childs-Disney *et al.*, 2007; Guan *et al.*, 2012) used aminoglycoside microarrays to study selected RNA internal loop-ligand interactions. This study attracted more attention (Aminova *et al.*, 2008; Tran *et al.*, 2010; Tran *et al.*, 2011; Velagapudi *et al.*, 2013) to utilize two-dimensional combinatorial screening (2DCS) to search the modified aminoglycosides that bind to the small RNA internal loops. In 2DCS, the interactions of aminoglycosides and RNAs were screened in a library-versus-library screening manner. The RNA internal loops that can bind to derivatives of neamine, tobramycin, kanamycin A and neomycin B were then identified by 2DCS platform. The results suggested that 2DCS is a unique platform, which can determine RNA and chemical space simultaneously and also identify certain interactions between RNA motif and ligand. This information may provide great potential to the rational and modular design of small molecules targeting RNA. A number of amino glycosides can be tested in parallel in a single test with minuscule amount of testing samples and RNAs by the micro array technology, which is much more efficient compared with traditional methods. It also suggested that the amino glycoside micro arrays play an important role in the discovery of new RNA targets.

RNA molecules are important therapeutic targets in the drug discovery process. To discover new RNA-binding molecules, small molecule microarrays have been used to screen from a large unbiased library of drug-like small molecules (Foong *et al.*, 2012; Noblin *et al.*, 2012; Sztuba-Solinska *et al.*, 2014; Singh *et al.*, 2015). Micro arrays provide a valuable opportunity to explore molecular interactions between drug and RNA targets in a high-throughput manner. The small molecule microarrays results will accelerate the identification of novel RNA-binding molecules.

Interactions with toxicity- and resistance-causing enzymes

The usages of aminoglycosides are limited due to their inherent toxicity and the increasing resistance of aminoglycosides caused by toxicity- or resistance-causing enzymes. Thus, the ideal antibiotics should have satisfactory antimicrobial activity with low toxic and side effects, and avoid development of resistance. Microarray technology was also used to identify the interactions of antibiotics with these enzymes.

Two types of toxicity-causing enzymes, Klenow DNA polymerase from *Escherichia coli* (Ren *et al.*, 2002) and phospholipase C from *Bacillus cereus* (Morris *et al.*, 1996), are common models for study of aminoglycosides toxicity. The interactions of aminoglycosides with Klenow DNA polymerase and phospholipase C may cause side effects, such as ototoxicity and nephrotoxicity. In order to reduce those side effects to the greatest extent and improve the therapeutic effects, aminoglycoside microarrays provide a powerful tool to identify novel antibiotics that do not bind or bind weakly to toxicity-causing enzymes.

Bacterial resistance to aminoglycosides is usually caused by resistance-causing enzymes, such as AACs, ANTs and APHs. These resistance enzymes in Gram-positive pathogens are APH (3')-IIIa and AAC (6')-Ie/APH (2")-Ia (Miller *et al.*, 1997). Three kinds of AACs resistance enzymes, AAC (2') from Mycobacterium tuberculosis (Magnet *et al.*, 2001), AAC (6') from Salmonella enterica (Hegde *et al.*, 2001) and AAC (3) from *Escherichia coli* (Magalhaes *et al.*, 2005), could be modified on A-site binding site to influence the therapeutic efficacy of aminoglycosides. These three kinds of AACs resistance enzymes were incubated with aminoglycoside microarrays to study their binding capacity and enzymatic activity (Disney *et al.*, 2004; Barrett *et al.*, 2008; Disney *et al.*, 2007). In the study of Seeberger and his colleagues, the microarray results showed that all immobilized aminoglycosides could interact with AAC (2') and AAC (6') enzymes after hybridization, which correlated well with a reported study of binding affinity (Disney *et al.*, 2004). AAC (2') and AAC (3) enzymes were also found to bind with the microarrays (Barrett *et al.*, 2008). Those results suggested that acetylation modification of the 2'-NH_2 had a minimal effect on A-site binding compared to modification at other positions directly contact the RNA, such as the 2"-NH_2, 3-NH_2, or 6'-NH_2. It has been reported that the binding of APHs and ANTs with aminoglycoside could also be identified by microarrays (Disney *et al.*, 2007; Chevolot *et al.*, 2012). Furthermore, the modification of aminoglycosides by both APH (3') and ANT (2") decreased the binding affinity of the aminoglycosides with their rRNA targets, which was consistent with the results of solution measurements (Ding *et al.*, 2003; Shandrick *et al.*, 2004; Verhelst *et al.*, 2004).

Discovery in inhibitors of resistance-causing enzymes

There are several mechanisms for bacteria achieving resistance to aminoglycosides, such as enzymatic modification, modification of bacterial rRNA leading to disruption of hydrogen bonding to decrease affinity with aminoglycoside, and changing the composition of cell membrane to reduce the drug uptake (Beauclerk *et al.*, 1987). It has been reported that *O*-phosphorylation, *O*-nucleotidylation and *N*-acetylation can decrease the binding affinity of aminoglycosides with rRNA A-site (Llano-Sotelo *et al.*, 2002; Barrett *et al.*, 2008).

Guanidinoglycosides, a series of designed aminoglycoside analogues, were arrayed onto derivatized glass slides to search inhibitors of resistance-causing enzymes (Disney *et al.*, 2004; Fair *et al.*, 2012). Due to the difference between the dissociation constants of guanidine and amino groups (12.5 versus 8.8, respectively), guanidinoglycosides may not be the suitable substrates for AAC (2') and AAC (6'). And the guanidinoglycosides possess a stronger binding affinity towards the negatively charged binding position in AACs due to its increased positive charge. The ability of guanidinoglycosides as substrates for AAC (2') and AAC (6') was tested by a spectrometric assay (Magnet *et al.*, 2001; Hegde *et al.*, 2001). The results revealed that guanidinoglycosides exhibited stronger affinity to AAC (2') and AAC (6') compared with aminoglycosides without modified guanidine group (fig. 5). It was also indicated that guanidinoglycosides are the inhibitors of acylation of several clinically used aminoglycosides but not the substrates for AACs enzymes (Morris *et al.*, 1996). Only kanamycin A and tobramycin derivatives (3') had been reported as inhibitors of ANT (2") and APH. Since kinase has been successfully screened by microarrays, it is promising to identify novel APHs or ANTs inhibitors (Goldstein *et al.*, 2008; De Pascale *et al.*, 2011) by the microarrays technique.

CONCLUSIONS

In a word, aminoglycoside microarrays had emerged as a powerful platform over the last decade. The outstanding advantage of aminoglycoside microarrays is that it can screen large numbers of aminoglycosides in parallel with tiny amounts of analyte and ligand. The common way to fabricate aminoglycoside microarrays is a site-specific and covalent immobilization. The aminoglycoside microarrays can be well applied in development of novel antibiotics with strong binding affinity to RNA targets and causing low toxicity and resistance, discovery of new RNA targets, and identification of the resistance-causing enzymes inhibitors.

Obviously, the study of aminoglycosides in biology and biomedicine is of significant importance. Aminoglycoside microarrays technology will be widely used for high throughput screening. There are still some challenges in the development of aminoglycoside microarrays. Firstly, the discovery of novel aminoglycosides is hampered by the tedious and low-yield isolation of natural aminoglycosides. The improvement of the yield of fermentation of bacterial genera by novel biosynthetic pathways and development of better isolation methods will be critical ways to discover new aminoglycosides. Secondly, novel methods for immobilizing

aminoglycosides to the slides are urgently to be explored and improved. Finally, aminoglycoside microarrays technology is only primarily used in a small quantity of laboratories around the world. With the rapid development of biology and biomedicine, aminoglycosides microarrays will come into a mature field, as well as DNA microarrays and protein microarrays.

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