·综 述·

纳米抗体筛选技术研究进展

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摘 要：纳米抗体(nanobody, Nb)是在骆驼科血清中发现的一种新型抗体，具有体积小、特异性强、稳定性高、易于表达和能识别隐藏的抗原表位等优势，在各个领域具有广泛的应用价值。本文介绍了纳米抗体筛选与优化过程，包括纳米抗体文库构建、体外展示和亲和力成熟 3 个重要技术阶段的分类与特点。其中，简要描述了天然、免疫及半合成/合成文库的制备方法与重要参数，并系统介绍了应用噬菌体、酵母、细菌、核糖体/mRNA 和真核细胞等表面展示系统，以及酵母双杂交、高通量测序和质谱鉴定方法，共 8 种不同体外展示技术进行快速筛选的方法及其优缺点，汇总用于提升纳米抗体功能可靠性的体外及计算机辅助亲和力成熟技术平台，为综合运用各种技术手段快速获得稳定、可靠、特异的纳米抗体类药物或诊断制剂提供了参考。

关键词：纳米抗体；体外展示筛选；亲和力成熟

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Advances in nanobody screening technology

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Abstract: Nanobody (Nb) is a novel type of antibody discovered in the serum of Camelidae. It is characterized by its small size, high specificity, stability, and ease of preparation. Nanobodies exhibit the ability to identify hidden epitopes and have diverse applications across various fields. This review aims to introduce three key stages in the screening and optimization of nanobodies, including nanobody library construction, *in vitro* surface display, and affinity maturation. We provide a brief description of preparation and characteristics of natural, immunological, and semi-synthetic/synthetic libraries. Additionally, we systematically explain eight *in vitro* display methods, including phage display, yeast display, bacterial display, ribosome display/mRNA display, and eukaryotic cell display. Furthermore, we discuss the application of yeast two-hybrid system high-throughput sequencing and mass spectrometry identification. A thorough analysis of their advantages and limitations is presented in this protocols. Finally, we summarize the platforms for *in vitro* or computer-aided affinity maturation techniques aimed at enhancing the functional stability of nanobodies. Consequently, this review provides a comprehensive approach to the integrated utilization of various technologies for the rapid development of stable, reliable, and specific nanobody-based drugs or diagnostic agents.

Keywords: nanobody (Nb); *in vitro* display screening; affinity matures

纳米抗体(nanobody, Nb)，即重链单域抗体(variable domain of heavy chain of heavy-chain antibody, VHH)，其发现于 1993 年，晶体直径 2.5 nm，长 4 nm，轻链天然缺失，分子量为 12−15 kDa，是自然存在可以和抗原结合的最小片段[1]。与常规抗体及抗体片段相比，VHH 具有较长 CDR3 区域，组成了抗原决定簇的主要部分，由于其独特的结构和更小的尺寸，可结合到折叠蛋白的平面、表面和裂缝等常规抗体难以企及的位点[2-3] (图 1)。此外，VHH 能耐受极端的温度和 pH、有机溶剂[4]和蛋白酶[5]等。经过热变性和化学变性后，VHH 可以对 CDR1 和 CDR3 之间的二硫键进行重新折叠和改造，有助于提高结构稳定性，保证其生物活性。同时，对高温的耐受有利于通过提高温度来增强生物识别的动力学[4-6]。Nbs 还是多聚化的理想候选抗体类型，可实现多价、多副表位和多特异性工程化改造，便于增加亲和力、结合多种抗原或体外亲和力成熟[7]，其简单的分子结构更容易在不同表达系统中大量表达，例如细菌、酵母或哺乳动物细胞[8-9]，成为适合进行工程化、规模化生产的潜在抗体类型。

基于以上特点，Nbs 已被广泛用于科学研究和临床诊治，包括正电子发射断层显像(positron emission tomography, PET)成像诊断、嵌合抗原受体 T 细胞(chimeric antigen receptor

T-cell, CAR-T)治疗、抗病原微生物疗法以及实体癌和血液癌的治疗等领域，成为基础研究和生物医学中一类引人注目的新型抗体[10-12]。为实现在不同疾病和领域的应用，开展纳米抗体筛选技术研究将有助于加速抗体的获得，而文库构建、体外展示和亲和力成熟作为纳米抗体筛选技术的 3 个重要阶段，则是完成高效、快速筛选技术的关键步骤，因此，本文对 3 种类型文库——免疫文库、天然文库和半合成/合成文库进行总结，并重点介绍了这 8 种不同纳米抗体库的筛选技术(图 2)，结合亲和力成熟技术，有望解析纳米抗体筛选 3 个关键步骤的技术特

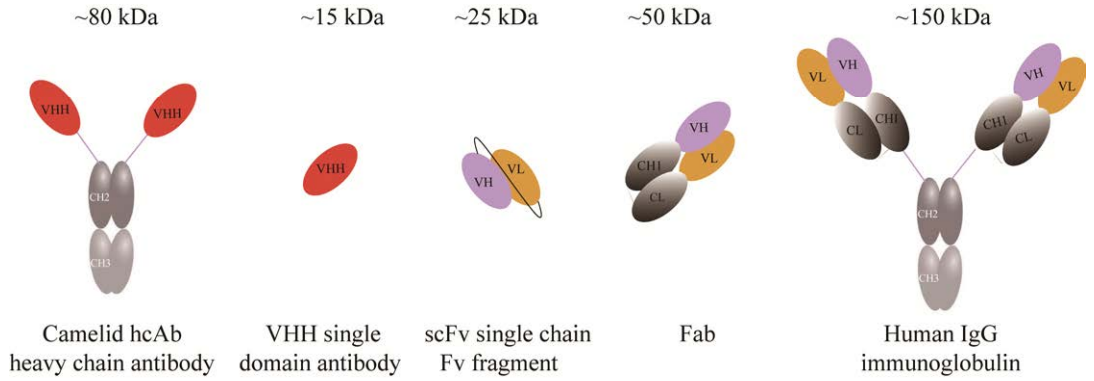

图 1　不同形式抗体结构示意图

Figure 1　Schematic diagram of the structure of different forms of antibodies.

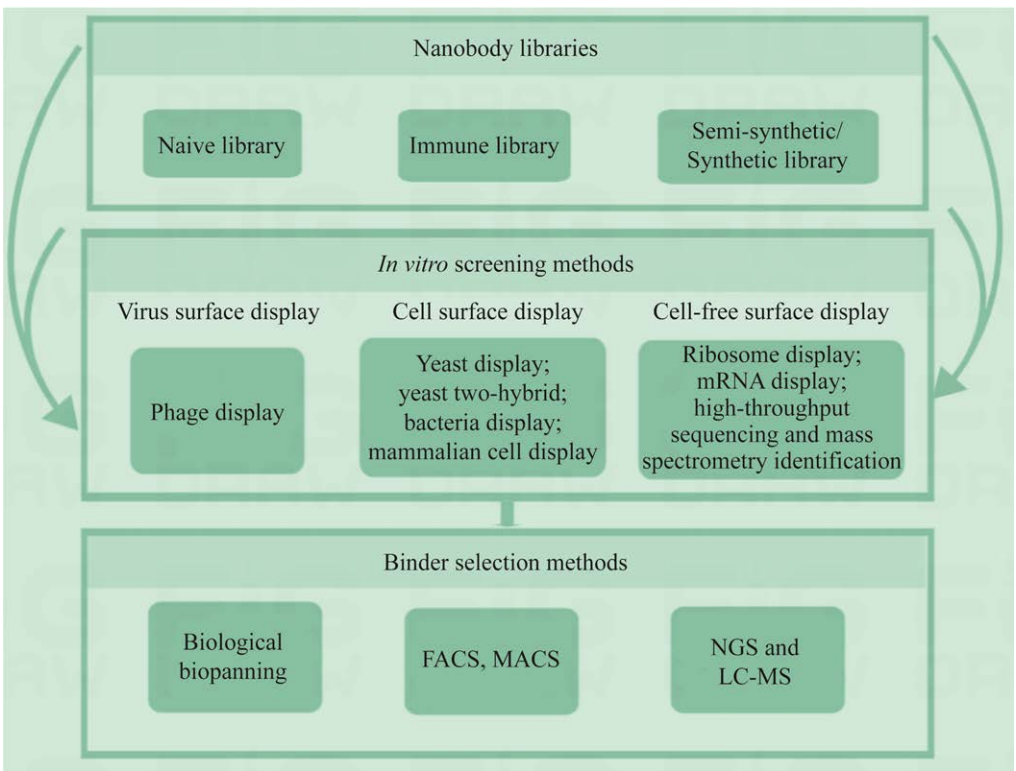

图 2　文库、表面展示及筛选系统的类型

Figure 2　Types of libraries, surface displays and screening systems.

点，促进高效筛选策略的发展，以期为相关研究领域提供技术支持。

1 纳米抗体库的分类及特点

1.1 天然库

在天然文库中，收集多个健康、未免疫动物的血液样本分离总淋巴细胞，构建天然文库。然而，采集不同个体的大量血液(通常超过 1 L)是确保天然文库多样性的先决条件，天然文库无抗原偏好性，可以用于任何潜在抗原筛选。由于利用宿主免疫系统的高度多样性，天然文库避免了免疫步骤。由于缺乏体内免疫亲和力成熟步骤，因此需要大量的血液才能产生较大的文库大小，所以只有从库容大、多样性高的文库中筛选时才能实现高特异性和亲和力，或需要亲和力改进步骤来获得高结合亲和力 Nb[13-14]。此外，对于大多数生物实验室而言，骆驼科动物是很难获得的。

1.2 免疫库

骆驼科动物是纳米抗体文库的主要来源之一，动物免疫可获得亲和力低至 nmol/L 或 pmol/L 水平的特异性单域抗体[15]。免疫库首先要制备抗原，经过 3–4 轮免疫后，获取免疫动物的 B 淋巴细胞，构建针对某个抗原的特异性免疫库。VHH 基因一旦被扩增并构建至载体中，便可利用噬菌体展示技术、细胞表面展示或者可基于 mRNA/核糖体展示或质谱鉴定等方法分离高亲和力的 Nbs。免疫库的优势是可以选择具有高亲和力、特异性和功能性的结合剂，避免了进一步抗体成熟的必要性。同时，其缺点包括：不同抗原需要多个文库；非免疫原性抗原或对宿主有不利影响的抗原，可能无法诱导免疫反应；以及该方案非常昂贵且耗时，短时间内较难达到理想结果[16]。因此，半合成/合成文库可能是更实用的替代方案，用于任何潜在抗原的筛选。

1.3 合成库与半合成库

半合成文库是天然序列与人工合成的一部分可变区序列组合而构建，而全合成文库的框架区以及可变区则全部由人工合成。构建合成与半合成文库时，应确定框架序列，cAbBCII10 被认为是合成文库支架的良好选择[17]，其框架序列稳定、表达良好且具有高度通用性。其次是互补决定区(complementarity determining region, CDR)的随机化设计，可变区域的多样性和有效性之间的平衡是首要考虑因素，基于决定亲和力和特异性的 CDR3 主要区域或同时基于 3 个 CDR 的随机化来设计文库，天然纳米抗体的 CDR3 长度为 3−28 个氨基酸不等，实现随机化的最简单方法是使用简并密码子 NNN 或 NNK (其中 N=A/T/C/G，K=G/T)，便可以覆盖所有 3 个氨基酸或者根据天然抗体 CDR 氨基酸组成偏好，或通过三聚体密码子(trimer codon)等技术合成[18]。虽然合成与半合成纳米抗体文库可省去动物免疫周期，直接用于纳米抗体的筛选，但关键是对 CDR 区进行大型而多样化的合理化设计以产生更多的 CDR3 多态性才能满足文库多样性的需求[19](表 1)。

2 纳米抗体体外展示筛选方法以及优缺点

2.1 噬菌体展示

噬菌体是在细菌内感染和复制的一种病毒，丝状噬菌体将单链 DNA 注入靶细菌细胞，随后在宿主细胞质内复制和组装新的病毒粒子。由于其易于操作和在各种温度、pH 值下的稳定性，可作为重组技术可靠的载体[26-29]。

噬菌体展示已成为体外抗体开发中使用最广泛的系统。George P. Smith 于 1985 年第一个

表 1 合成与半合成文库框架及随机区域设计

Table 1 Synthetic and semi-synthetic library framework and randomized region design

Framework	Randomized region	Randomized region design	Biopanning method	Capacity size	Application	References
cAbBCII10	CDR3 only	NNK (where N=A/T/C/G, K=G/T)	Phage display	1.6×10^{9}	Successfully screening Nb targeting human prealbumin (PA) and neutrophil gelatinase-associated lipid carrier protein (NGAL) for the development of a highly sensitive and specific PA detection system	[20]
cAbBCII10	Three CDRs	NNK	Phage display	2.6×10^{9}	Successfully screening Nb targeting phosphatidylinositol proteoglycan 3 (GPC3) as a potential diagnostic and therapeutic molecule for HCC	[21]
cAbBCII10	Three CDRs, and the length of CDR3 (9–20 amino acids)	Degenerate codon synthesis, where CDR1 and CDR2 regions are partially randomized and CDR3 is fully randomized	Phage display	1.0×10^{12}	Successful identification of Nb targeting the M2 ion channel protein of influenza A virus was achieved, demonstrating potent neutralizing activity against influenza A virus	[22]
Humanized nanobody scaffold hs2dAb	Three CDRs	Trimer synthesis; CDR1 and CDR2 regions were partially randomized, while CDR3 was completely randomized except for cysteine	Phage display	3.0×10^{9}	Fluorescent protein, actin, tubulin, and high-affinity binding agents have been successfully screened	[23]
Alpaca IGHV3S53 for the framework	Three CDRs	Degenerate codon synthesis, three CDRs, and length of CDR3 (6−18 amino acids)	Phage display	1.0×10^{9}	Successfully screened Nb, which targets the extracellular domain of soluble mouse programmed cell death protein 1 (PD-1), and inhibited the interactions between mPD-1 and mPD-L1	[24]
Three framework from the PDB database source: 3K1K, 3P0G, 1ZVH	Three CDRs partial randomization, where CDR3 corresponds to lengths of (6,12,16 amino acids)	Trimer synthesis to obtain a balance between charged, polar, aromatic and non-polar amino acids	Ribosome display and phage display	9.0×10^{12}	The transient conformations of Nb-targeting maltose binding protein (MBP), bacterial ABC transporters IrtAB and TM288/288, human solute carrier (SLC) transporter ENT1, and GlyT1 membrane protein were successfully screened	[25]

引入噬菌体展示[30]。自 1990 年以来，噬菌体展示是用于分离靶标特异性纳米抗体的最广泛和标准的选择技术[31-33]。噬菌体展示技术是将外源基因与噬菌体载体 pIII 蛋白基因进行融合表达，并随噬菌体组装展示在其头部，连接基因型与表型，是一种强大且实用的蛋白质分子相互作用研究工具[34]。噬菌体展示技术通常涉及捕获靶抗原，通过固定在固相载体表面上或与磁珠偶联。通过多次洗涤去除非结合的噬菌体，而附着的噬菌体通常在低或高 pH 条件下洗脱以破坏抗体-抗原结合，用于后续几轮淘选扩增。由于非特异性结合的存在，至少要进行 2−3 轮筛选，以富集正结合剂并减少非特异性结合。目前，虽然噬菌体是最常见的展示系统，但也有研究者采用了如下替代系统，例如核糖体展示和细胞展示，包括酵母和细菌展示，以及哺乳动物细胞展示，每一种文库以及每一种展示方法具有一些优点和缺点，这些展示系统的复杂性各不相同。

2.2 酵母展示

1997 年引入了酵母展示方法[35]，在酵母表面上展示外源蛋白是通过与酵母细胞壁蛋白融合来实现的。在大多数酵母展示系统中，展示在酵母细胞壁上的大多数异源蛋白通过其 C 末端的糖基磷脂酰肌醇(glycosylphosphatidylinositol, GPI)锚定共价连接到 β-1,6-葡聚糖[36]。GPI 锚定蛋白用于展示异源蛋白，包括 Aga1p、Aga2p、絮凝素等。GPI 锚定的蛋白质通过内质网-高尔基体分泌途径转运到酵母细胞表面，并与细胞壁甘露蛋白层形成 β-1,6-葡聚糖桥[37]。目前，基于酿酒酵母 Aga1-Aga2 的酵母展示系统是最受欢迎的系统[38]。其中，Aga2p 融合蛋白被编码在工程质粒中，而 Aga1p 被编码在酵母基因组中。两者都在半乳糖诱导的启动子 GAL1 的控制下表达，二硫键的形成确保了 Aga1p 和 Aga2p 蛋白的组装。该载体具有血凝素标签(hemagglutinin tag, HA)和 C 末端的 c-myc 标签，针对这些标签，可以基于流式细胞术进行筛选[39-40]。此外，一种或两种外源蛋白可以展示在与酵母 Aga2p 的 C 端或 N 末端融合[41](图 3)。

酵母表面展示是一种可靠的方法，具有可溶性高易于筛选等特点，Ryckaert 等[42]用绿色荧光蛋白(green fluorescent protein, GFP)免疫的美洲驼文库通过融合到 α-凝集素的 C 端结构域展示在毕赤酵母的表面上，筛选到 GFP 特异性 Nb 具有亲和力常数(K_D)在 nmol/L 范围的亲和力。

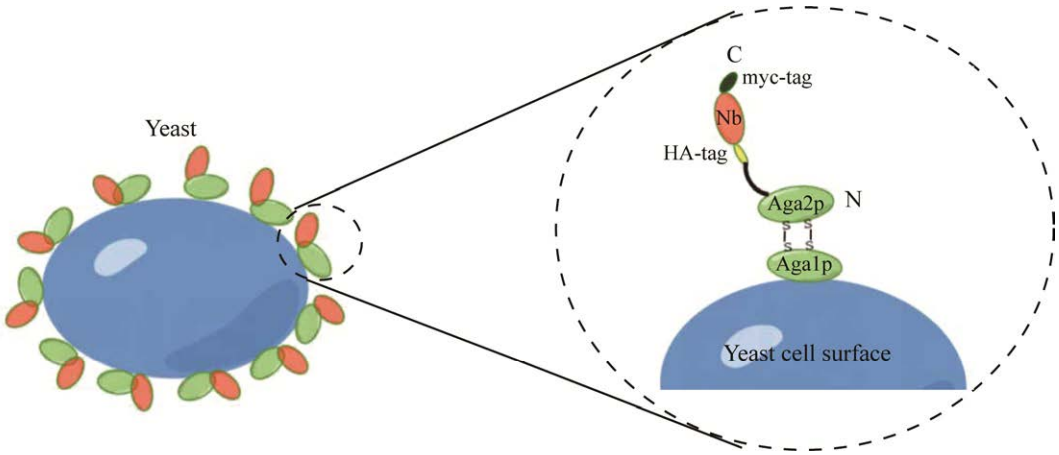

图 3 酵母展示示意图

Figure 3 Schematic diagram of yeast display.

2.3 酵母双杂交

酵母双杂交系统是迄今为止用于鉴定蛋白质-蛋白质相互作用最广泛使用的选择技术，该系统于 1989 年开发[43]。它在真核生物转录调控中建立，真核生物有 DNA 结合结构域(DNA-binding domain, DNA-BD)和转录激活结构域(transcription-activating domain, DNA-AD)，可以特异性结合到基因上游的转录起始点。BD 和 AD 单独作用并不能激活转录反应，当二者在空间上充分接近时，才能激活下游启动子使下游基因得到转录[44]。在酵母双杂交中，需要把待测目的蛋白分别构建至两种载体中，与 DNA-DB 和 DNA-AD 融合的蛋白称作诱饵蛋白(bait)和猎物蛋白(prey)，相应的载体称作 bait 和 prey 载体，通过对报告基因的检测，可判断两者之间是否存在相互作用[43]。同时根据酵母有性生殖特点，将文库分别构建到 bait 和 prey 载体上，转化到不同生殖型的酵母中进行交配，形成的二倍体细胞便表达了不同的诱饵和猎物蛋白，从而能够筛选细胞内所有相互作用的蛋白。

基于酵母双杂交技术是研究蛋白相互作用的有效工具，可以将抗原蛋白构建到诱饵质粒融合表达，避免了耗时费力的抗原制备纯化过程，同时在未制备抗原的条件下，Gao 等[45]构建了新城疫病毒(newcastle disease virus, NDV)免疫的 VHH 酵母双杂交文库，首次筛选到了 7 个靶向新城疫病毒血凝素-神经氨酸酶(haemagglutinin-neuraminidase, HN)蛋白的 Nbs，并可能在 NDV 的诊断、治疗和发病机制中显示出巨大的应用潜力。

2.4 核糖体和 mRNA 展示

近年来，核糖体和 mRNA 展示已被证明是非常成功和实用的体外展示技术，用于筛选具有大型文库的功能肽和蛋白质。首先，核糖体展示[46]包括一个简短的体外翻译反应，在适当的条件下停止，对于核糖体展示，文库的两侧必须有一个上游启动子区域和下游携带无终止密码子的间隔区域。DNA 文库首先在体外转录成 mRNA，然后通过核糖体翻译成蛋白质，从而连接表型和基因型，以生物素化形式为靶标，并通过链霉亲和素包被的磁珠捕获核糖体复合物，并洗涤以去除弱结合或非特异性结合的文库组分。mRNA 展示技术[46]，DNA 文库首先在体外转录成 mRNA，小分子嘌呤霉素连接到体外转录的 mRNA 上并进行体外翻译，嘌呤霉素模拟酪氨酰-tRNA 的结构功能进而与核糖体 A 位点结合，抑制蛋白质翻译，因此新生的多肽被转移到嘌呤霉素分子上，共价连接的 mRNA-蛋白质复合物被分离、逆转录并用于筛选实验。通过在高 pH 下水解互补的 mRNA 从靶标结合的复合物中回收 DNA 链用于 PCR 扩增(图 4)。核糖体和 mRNA 展示完全在体外进行，克服了基于细胞展示系统的局限性，因此可构建出 10^{12}–10^{14} 高多样性的文库，通过引入突变产生更多的高亲和力抗体，是一种筛选大型文库和获取分子进化强有力的方法。

运用核糖体展示技术来靶向构象特异性膜蛋白的纳米抗体的筛选，Zimmermann 等[25]成功选择了针对细菌 ABC 转运蛋白 TM287/288 的瞬时 ATP 结合状态的 Nb 和针对人的 SLC 转运蛋白 GlyT1 和 ENT1 的构象选择性 Nb。因此，利用核糖体展示技术来筛选结合膜蛋白抗体具有良好效果。

2.5 细菌展示

大肠杆菌具有明显的优势，但针对 Ab/Nb 片段开发有效的大肠杆菌展示系统并不容易。因为大肠杆菌是一种具有细胞质内膜(inner membrane, IM)和外膜(outer membrane, OM)的革兰氏阴性细菌[47-48]。OM 的存在一直是阻碍开发大肠杆菌表面展示的主要障碍。为了展示在细

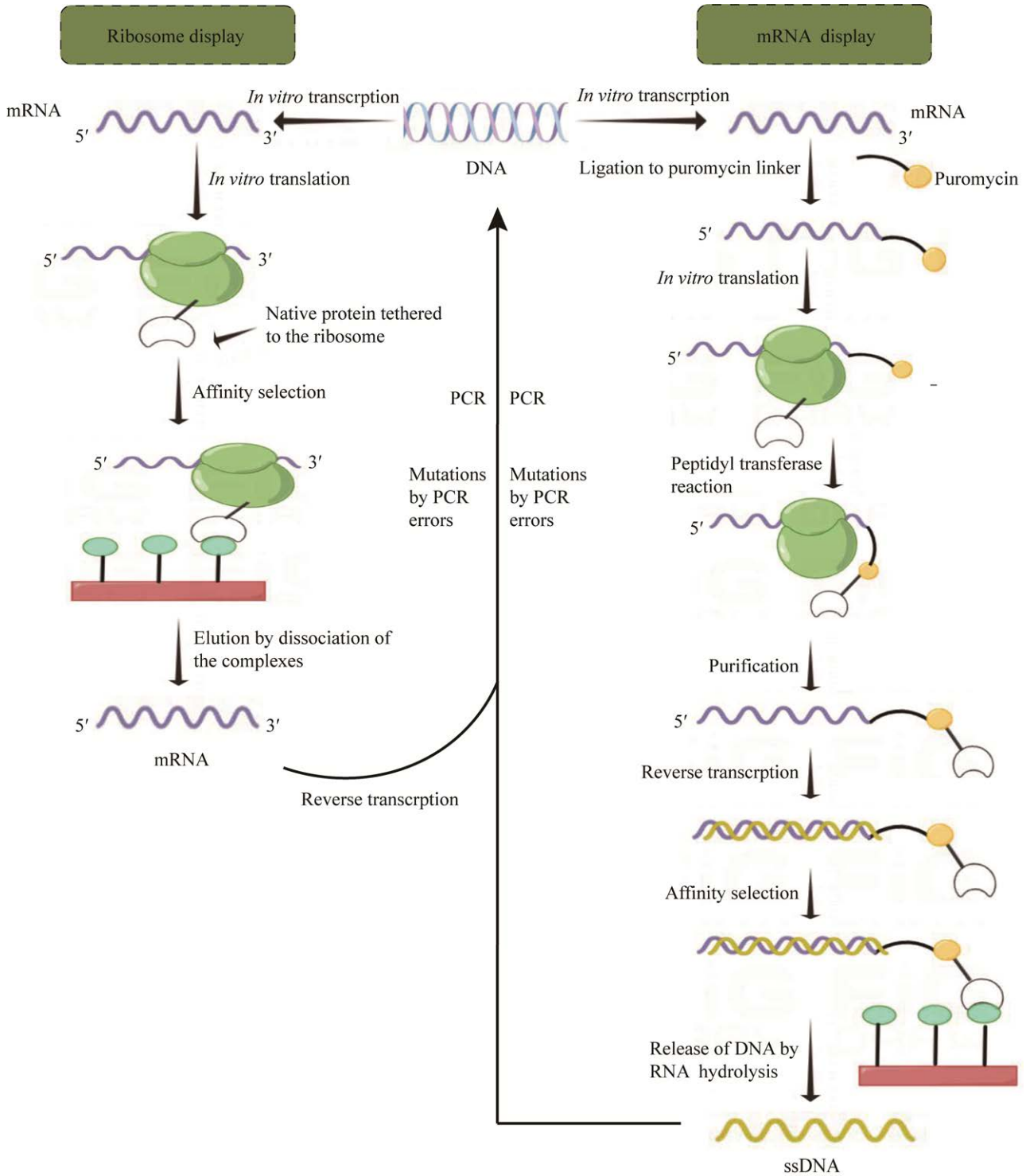

图 4　核糖体和 mRNA 展示示意图

Figure 4　Schematic diagram of ribosome and mRNA display.

菌表面，细胞质中产生的多肽必须通过 IM 移位，穿过周质，插入 OM 并将展示的多肽区域暴露在细胞外环境中，但通过对革兰氏阴性菌分泌的一大类蛋白质研究发现，自动转运蛋白(autotransporters, ATs)[49]可充当“辅助”结构域，协助多肽 N 末端部分跨 OM 移位，其中 AT 在其序列中包含 C 端 β 桶(β-barrel)结构，将多肽与保守的 β-barrel 组装成 BAM 复合物，BAM 复合物负责内膜 β-barrel 在 OM 中的折叠和插入，并参与 Ig-like 结构域向大肠杆菌细胞表面移位以展示和选择大肠杆菌中的 Nb 文库[50]。Salema 等[51]构建了用于大肠杆菌 Nb 表面展示平台的 pNeae2 质粒载体，包括 N 端信号肽区域和用于 OM 锚定的 β-barrel 和 Ig-like 结构域，该载体还携带 *lacZ* 启动子基因以及 VHH 两侧的表位标签(E 标签和 myc 标签)，用于检测融合蛋白在大肠杆菌表面上的表达情况，并通过磁珠分选选择高亲和力的 Nbs。

此外，使用此方法，Salema 等[52]利用大肠杆菌表面展示的优势特性以及 Nbs 在肿瘤治疗和体内肿瘤成像中的生物医学潜力，进一步评估大肠杆菌表面展示以筛选针对肿瘤相关细胞表面抗原的 Nbs，并利用肿瘤相关细胞表面抗原表皮生长因子受体(epidermal growth factor receptor, EGFR)构建大肠杆菌表面展示平台，结果表明大肠杆菌展示系统能够有效分离针对 EGFR 不同表位的高亲和力 Nbs。

2.6 哺乳动物细胞展示

由于病毒(噬菌体)、原核生物(细菌)或低级真核生物(酵母)等生物体具有与哺乳动物细胞不同的翻译后修饰功能，相比之下，哺乳动物细胞包含完整的糖基化修饰、蛋白质折叠功能，是治疗性抗体筛选的理想展示系统[53-54]。然而，将高多样性 VHH 文库引入哺乳动物细胞也具有挑战性。Zhao 等[55]使用简并引物(NNK)将随机 CDR 序列引入 dsDNA 开发了两种在哺乳动物细胞中构建 Nb 的新方法：一是将含有重叠序列上下游 dsDNA 共转染至哺乳动物细胞进行细胞内同源重组，从而形成完整的 Nb 文库表达框；二是在体外采用酶切连接的方式生成全长序列转染至细胞中用于文库表达。并通过高通量以及单细胞测序来解析纳米抗体文库的多样性。由于哺乳动物细胞转染效率低、抗体易组装错误、轻重链表达不一致等因素导致库容多样性只有 10^6–10^8，且在建库过程中较难保证只有一种外源 DNA 进入单个细胞，可能会对后期筛选产生一定的干扰。但哺乳细胞表面展示系统可利用流式细胞荧光分选技术(fluorescence activated cell sorting, FACS)筛选、监测高亲和力抗体的生成，且可用于构建小分子和全长抗体库。因此，哺乳细胞表面展示仍然是体外筛选治疗性抗体的一个重要手段。

哺乳动物细胞是展示跨膜蛋白和作为细胞表型调节剂的理想平台。此外，Schmidt 等[56]还开发了一种人体细胞的慢病毒筛选方法，以鉴定抗病毒 Nb。哺乳动物细胞展示的 Nb 也发现了一些有趣的应用，例如它们用于修饰具有适合癌症免疫治疗的嵌合抗原受体(chimeric antigen receptor, CAR)的 T 细胞。VHHs 优于 scFvs，因为 VHHs 被认为免疫原性更低、更稳定且结构更紧凑[57-59]。

2.7 高通量测序和质谱分析

高通量测序和质谱鉴定技术避免了体外构建展示文库和筛选等过程，因而能够在较短时间内快速筛选到大量针对特定抗原多个表位的 Nb。Fridy 等[60]报道了一种策略，该策略允许快速产生针对选定抗原的高亲和力 Nb 的大库。该方法侧重于来自免疫美洲驼的骨髓淋巴细胞，获取 RNA 以产生 cDNA，通过巢式 PCR

获得 VHH 区域以进行高通量测序，同时在美洲驼免疫后的血清中对 HCAb 与常规抗体进行亲和纯化分离含 VHH 的 HCAb，进一步采用木瓜蛋白酶消化以切割恒定区域并保留了 VHH 区域所需的最小片段，并通过液相色谱-质谱进行分析。以创建用于质谱(mass spectrometric, MS)分析的可搜索肽数据库。并通过原核表达和表面等离子体共振或其他技术进一步评估其表达能力与亲和力，在未构建文库的条件下快速筛选到了针对 GFP 的高亲和力 Nb (表 2)。

3 纳米抗体体外亲和力成熟方法

3.1 亲和力成熟

在体液免疫中，再次应答所产生抗体的平均亲和力和特异性高于初次免疫应答，这一过程被称为亲和力成熟。在体内，亲和力成熟离不开两个过程，首先在骨髓抗体轻重链的胚系基因片段经 V-(D)-J 随机重排组合，其次是互补决定区 CDR 发生高频突变，滤泡树突状细胞进行高亲和力抗体筛选[65-66]。而来源于天然抗体库中的抗体亲和力水平仅与动物在初次免疫应答后获得的抗体亲和力水平相当，仅为微摩尔级别，因此人们模拟体内亲和力成熟原理及过程、采取各种策略对抗体基因进行相应突变，进行体外亲和力成熟研究。抗体体外亲和力成熟包括随机突变、定点突变以及高通量合成等多种方式，而 CDR 则是突变关键区域[67-70]。通过体外亲和力成熟技术可以提高抗体药物的亲和力，从而减少其注射剂量，降低副作用，降低治疗成本[71-72]。

3.2 多聚化与多特异性

由于 VHH 的高溶解度、单域和单基因性质允许使用基因编码的氨基酸接头或载体蛋白快速成功地生成多聚体 VHH，由于亲和效应、多聚化和多特异性是一种快速提高功能效力的简单方法。由于 VHH 结构简单，仅有一个结构域，可以通过插入几个氨基酸的柔性 Gly-Ser 接头聚合，然后转化为多价和多特异性形式。纳米抗体多聚化已被证明可以提高与靶蛋白的亲和力。Detalle 等发现三价 VHH 与其单价形式相比，对呼吸道合胞病毒(respiratory syncytial virus-subgroups A, RSV-A)的中和效力提高 6 000 倍，对 RSV-B 的中和效力提高了 1 000 倍[73-74]。Hmila 等[75]在小鼠实验中成功地得到了抗蝎毒 AahI/AahII 的双特异性 Nb，可以有效抵抗致死蝎毒，另外，该双特异 Nb 还具有极显著的中和蝎毒抗原的能力。

3.3 计算机辅助亲和力成熟

随着计算机生物学的快速发展以及各种算法优化和计算机能力的提升，基于三维结构的体外亲和力成熟策略得以迅速发展，此外，VHH 因短小序列极大地减少了合理优化其生物物理特性所需的计算时间，VHH 适合于 X 射线结晶学和核磁共振分析，用于模拟 3D 构象，可以有效地获得 VHH 的准确结构，并精确分析 VHH 与靶分子的相互作用，同时借助不同的算法可以更好地了解抗体-抗原的结构和相互作用并进一步分析参与分子间相互作用的残基，有助于研究人员选择适合诱变的候选残基，以快速提高亲和力[76-77]。目前，利用计算机辅助技术结合高通量筛选技术进行抗体的体外亲和力成熟策略被多次报道，Wang 等[78]利用同源建模和分子对接技术结合丙氨酸扫描及双位点饱和突变的策略筛选到针对霉菌赭曲霉毒素 A (ochratoxin A, OTA)的 Nb，其亲和力常数提高 1.36 倍。Sulea 等[79]开发了抗体和蛋白质治疗药物辅助设计(assisted design of antibody and protein therapeutics, ADAPT)平台应用于 VHH 体外亲和力成熟的研究，得到亲和力常数 K_D 为 2 nmol/L 的突变体，K_D 提高了 9.4 倍。

表 2 纳米抗体体外展示筛选方法的优缺点

Table 2 Advantages and disadvantages of nanobody *in vitro* display screening method

Display types	Advantages	Disadvantages
Phage display[61-62]	The most extensive and standardized selection technology has been developed, with a more mature process; the integration of protein/genotype and phenotype has been achieved; and the preparation is straightforward, characterized by a short cycle and low cost	The limited size of foreign proteins is demonstrated by their small volume; the absence of features such as post-translational modification results in exacerbated misfolding issues; and a substantial amount of electrical conversion is required to accumulate a diverse library
Yeast display[61-62]	Efficient post-translational modification mechanism; solubility enhanced; insertion of heteroprotein without damaging yeast surface protein structure and surface display efficiency; selection *via* FACS and MACS technology	Low eukaryotes, various biological functions are difficult to compare with higher karyotes. Secondly, yeast cell transformation efficiency is low
Yeast two-hybrid[44]	Easily manipulable, plasmid retrieval is straightforward, featuring nutritional markers and well-characterized reporter genes; the investigation of protein-protein interactions boasts high sensitivity and simplified operation procedures	To establish the self-activating activity and toxic effects of the decoy protein is crucial to avoid false positive or negative outcomes. Moreover, the scarcity of high-quality libraries remains a principal limiting factor
Ribosome display and mRNA display[46,63]	Larger libraries can be effectively visualized and challenging targets can be efficiently screened. mRNA display is achieved through a small cohesion molecule, enabling the formation of a covalent bond between mRNA and protein, thereby rendering it relatively stable	The formation of ternary complexes between RNA molecules and ribosomes occurs through non-covalent interactions under unstable conditions. The absence of cellular participation throughout this process results in an inability to undergo normal processing and modification following protein translation, thereby directly influencing the display effect
Bacteria display[51,61]	The microorganism in question is the most widely utilized for cloning, amplification, and large-scale protein expression, boasting a high conversion efficiency. It exhibits a relatively robust tolerance to mechanical stress and has a larger volume than phages. Consequently, it facilitates direct selection, screening, and characterization *via* flow cytometry-based methods	*Escherichia coli* is a bacterium characterized by its inner and outer membranes, which are surrounded by a thick cell wall. The presence of the outer membrane primarily impedes the surface display of *E. coli*
Mammalian cell display[64]	Presenting diverse post-translational modification functions compared to prokaryotes and lower eukaryotes, it serves as an optimal platform for displaying transmembrane proteins and exhibits reduced immunogenicity in cancer therapy. Its selection and screening can be effectively carried out using flow cytometry and immunomagnetic beads	The diversity of the display antibody library is limited, significantly lower than that of the phage library. Moreover, the operation is intricate, the process is protracted, and the cost is comparatively elevated
High-throughput sequencing and mass spectrometry identification[60]	This approach circumvents the need for *in vitro* library construction and screening, enabling rapid identification of numerous Nb epitopes specific to a particular antigen within a brief period, with a high success rate	Specific antigen-based animal immunization is indispensable, as are peptide databases. However, the costs associated with high-throughput sequencing and mass spectrometry identification are substantial

4 小结与展望

纳米抗体因具有相对较小的尺寸、单体行为、高稳定性、高溶解度、强大的穿透性、低免疫原性、生产周期短，以及结合常规抗体无法获得的表位能力，使纳米抗体成为许多治疗和生物技术应用的理想选择[80]。此外，目前已有大量基于多价、多特异性纳米抗体及其修饰结构的生物制剂在免疫性疾病、肿瘤、神经系统疾病、血液病和传染病等疾病领域进入了不同临床研究阶段[81-85]。目前，基于多特异性 Nb 结构开发的 SARS-CoV-2 中和抗体已进入不同的临床阶段，以克服病毒突变引起的耐药性，从而削弱变体的生存[86]。其中，Caplacizumab 是首个基于 Nb 的药物，用于治疗获得性血栓性血小板减少性紫癜的成人罕见的凝血障碍，为 Nb 在医学上的应用奠定了基础[87]；KN035 恩沃利单抗是全球首个用于免疫治疗的单域抗体且是目前唯一获批上市的皮下注射 PD-(L)1 抗体，用于治疗晚期结直肠癌[88]。虽然目前还没有其他基于 Nb 结构的药物被批准用于癌症治疗，但鉴于 Nb 在该领域的大量研究，可以预期未来将有更多的多聚纳米抗体分子进入后期临床试验。因此，用于分离大量高亲和力纳米抗体的快速而稳健的技术是广泛使用的重要工具。如前所述，上述介绍每一种文库以及每一种展示方法具有一些优点和缺点，在各种展示系统中差异较大，文库大小是成功展示技术的重要参数之一，目前尚无高效、单一的展示系统可以保证高产率的重组抗体的生产和筛选。这些展示方法仍有待进一步优化，未来将在治疗、研究和诊断市场中持续改进并发挥显著作用。

从抗体文库中筛选得到的抗体多种多样，抗体的亲和力和特异性差异会极大地影响抗体的应用，包括抗原结合、药代动力学和安全性等诸多方面。因此，体外亲和力成熟方法可以对其进一步优化以满足需求。虽然 Nb 结构简单，其多聚化和多特异性是一种快速提高功能效力的简单方法，但目前基于计算机辅助亲和力成熟技术引起了广泛关注，包括改善亲和力、特异性、热稳定性和人源化等。计算机辅助方法可以在短时间内经济高效地获得改良的突变体，借助不同的算法可以更好地了解抗体-抗原的结构和相互作用，以快速提高亲和力。

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