

小分子化合物非竞争免疫检测方法研究概述

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摘要: 农兽药、非法添加剂及其他有毒有害化学污染物在食品和环境中的残留引起人们越来越多的关注。这些残留物多为小分子化合物, 对其进行免疫测定因受限于分子量和抗原表位数量, 多用竞争法, 通常不能像检测大分子抗原一样采用夹心式的非竞争法, 这就制约了其检测的灵敏度、稳定性及工作范围等。但非竞争免疫分析法也并非完全不适用于小分子化合物, 这需要方法学上的改进或创新。作者从检测方法原理、体系构成及应用现状等方面综述了近几十年来有关小分子化合物非竞争免疫分析方法的研究成果, 以期为相关研究者提供借鉴和参考。

关键词: 小分子; 非竞争免疫; 半抗原

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A Review of Non-Competitive Immunoassays for Small Molecule Compounds

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Abstract: Pesticides, veterinary drugs, illegal additives and poisonous and harmful chemical pollutants in foods and environment have aroused more and more concerns. These residues are mostly assayed by competitive immunoassay modes due to their small molecular weight and single epitope. Since the sensitivity, stability, and liner range of competitive immunoassays are not as good as those in non-competitive sandwich immunoassays, methodological improvements could made non-competitive immunoassays suitable. Herein this review summarized their principles, elements and applications of non-competitive immunoassays for the detection of small molecules during last decades.

Keywords: small molecule compounds, non-competitive immunoassay, hapten

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小分子化合物(相对分子质量通常小于 1 000),如合成药物、甲状腺激素、小分子肽、环境激素和真菌毒素等,这类化合物在免疫反应中只具有反应原性,而没有免疫原性,通常在免疫化学上被定义为半抗原。由于分子体积和空间位阻的原因,小分子化合物通常只有一个抗原表位,免疫测定一般采用竞争性分析模式^[1-2]。在竞争性分析方法中,标记半抗原和抗体的量都是有限的,待测物与标记的半抗原竞争结合少量的固相抗体,反应平衡后,分离结合和游离的半抗原,最终读出信号强度与样品中待测物的含量成反比。竞争性免疫分析的灵敏度受抗体亲和常数的限制较大。小分子化合物的单克隆抗体的亲和常数在一般情况下很难超过 10^{12} mol/L,这就决定了竞争性分析模式难以达到亚飞摩尔浓度的测量水平。另外,在低浓度的反应信号很难与零区别开来,零剂量点的相对误差较大,分析过程的重现性差,反应孵育时间过长,所有上述因素致使竞争性免疫分析在灵敏度、精密性、动力学及工作曲线范围方面都不及非竞争免疫分析^[3]。

非竞争性分析方法目前主要用于大分子抗原的检测,最常用的是夹心法,使用两种不同的过量抗体,即固相捕获抗体和标记抗体,结合待测物,造成一种夹心式的分析模型。这种方法要求待测物分子必须是多价抗原,具有多个抗原表位,可以同时结合固相和标记抗体,然后检测标记抗体的活性以判断待测物的含量。这种检测模式显然不适用于小分子化合物。血管紧张素 II 是迄今为止采用双位点夹心法能够测量到的最小分子(相对分子质量 1 048)^[4]。反应体系中过量抗体的加入,使得非特异性信号成为影响非竞争性分析方法灵敏度的主要因素之一。如果抗体的亲和力足够高,降低非特异性反应,可以使夹心 ELISA 的最低检测限达到 $\text{atto mol}(10^{-18} \text{ mol})$ 分子水平^[5]。

关于小分子化合物非竞争性免疫分析方法的研究,近几十年来取得了一些重要的进展。一些新的检测原理和检测方法不断诞生,大大丰富了小分子半抗原的检测模式。

1 基于生物素亲和素体系的双位点夹心法

Ishikawa 等^[6]提出了基于生物素亲和素体系检测小分子肽的双位点夹心免疫分析模式,见图 1^[6]。

其检测原理是生物素化后的待测物可同时结合标记抗体和固相的亲和素,从而可建立针对半抗原的双位点夹心式的非竞争性分析方法。反应过程大致如下:首先,在待测物分子上引入生物素;生物素化的待测物经固相抗体纯化后,再与标记抗体混合;然后一同转移到预包被有亲和素的固相载体上,读出的信号强度与样品中待测抗原的含量成正相关。这种分析方法已经成功应用到血管紧张素 I^[6]、精氨酸加压素^[7]等小分子肽以及甲状腺素^[8]的测定。后来,Hashida 等^[9-10]又进一步发展了超灵敏的双位点复合物转移酶免疫分析法。通过多次免疫复合物的固相转移步骤,非特异性信号有了很大程度的降低,同时由于 4 种高亲和力固相抗体的浓缩富集作用,检测灵敏度也得到极大的提高。但这种方法过程较为繁琐,对反应试剂要求较高。

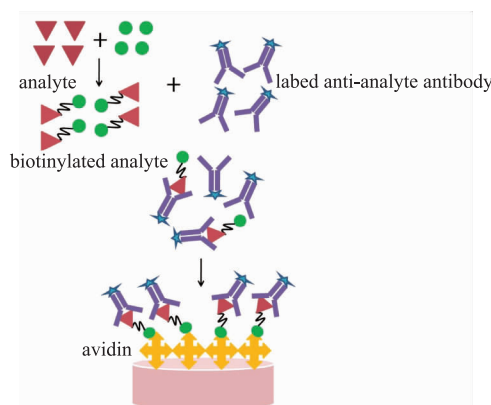


图 1 基于生物素亲和素体系的双位点夹心法原理模式

Fig. 1 Principle diagram of biotin and avidin based two-site sandwich immunoassay

2 基于固相固定化表位体系的免疫分析

Pradelles 等^[11-12]发展了基于固相固定化表位体系的非竞争性免疫分析方法 (Solid-phase Immobilized Epitope Immunoassay, SPIE-IA) 用于检测小分子半抗原。其反应程序见图 2^[12]: (a) 半抗原(标准品或样品)由固相抗体捕获; (b) 半抗原分子的氨基基团通过双功能试剂,如戊二醛或双琥珀酰亚胺辛二酸酯等,与固相蛋白发生化学交联; (c) 在酸、碱或有机溶剂的变性作用处理下,半抗原表位由抗体结合位点处释放; (d) 借助共价作用结合于固相载体上的半抗原,被释放的表位通过酶标抗体检测。这种分析模式的优点在于固相和标记抗体可

以是同一种抗体,省去了传统的双抗体夹心法需要筛选针对不同抗原决定簇的两种抗体的复杂程序;而局限性则在于要求目标分析物含有氨基官能团。该方法已被发展到甲状腺素^[11]、白三烯 C4^[13]、肿瘤转移抑制因子 metastin C 端结构域类似物 TAK-448 和 TAK-683^[14]等的测定。

当然,对于很多不含有氨基的半抗原分子,如促甲状腺激素释放激素(TRH),可通过化学修饰引入氨基基团,再进行 SPIE-IA 反应^[15]。也可以通过紫外辐照^[16]或类 Fenton 试剂产生的羟基自由基触发的方式^[17]实现半抗原与固相抗体的直接交联。

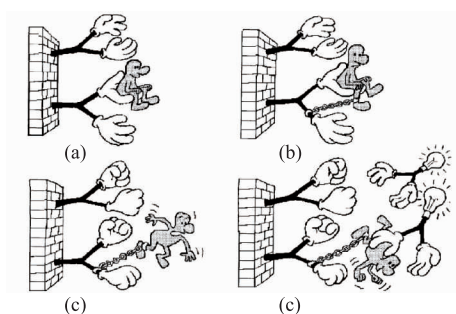


图 2 SPIE-IA 基本原理

Fig. 2 Principle diagram of SPIE-IA

3 基于抗独特型抗体的非竞争性免疫分析方法

抗独特型抗体 (Anti-idiotype Antibody, AId 或 Ab2) 是针对抗体 (Ab1) 可变区的抗原决定簇,即抗体的独特位,所产生的特异性抗体。独特型的差异,由抗体重链可变区 (VH) 和轻链可变区 (VL) 内氨基酸序列不同造成^[18]。抗独特型抗体主要有两类: Ab2 α 和 Ab2 β 。Ab2 α 识别抗体可变区骨架区内的独特型决定簇,是抗体中远离抗原结合部位的抗原决定簇产生的抗体,Ab2 α 与 Ab1 的结合不影响 Ab1 与抗原的结合,属半抗原非抑制性 Ab2; Ab2 β 是由抗体的抗原结合部位处的抗原决定簇产生的抗体,可完全抑制抗原与 Ab1 的结合,被认为是抗原的“内影像”^[19]。1990 年 Barnard 等^[20]报道建立了一种基于抗独特型抗体检测小分子化合物的非竞争性免疫分析方法,并用于血清中雌二醇的测定。其反应过程如图 3^[20]: (a) 将特异性抗体 Ab1 包被于固相载体,加入标准品或样品; (b) 加入 Ab2 β , 封闭 Ab1 中未被待测物占用的结合位点; (c) 加入标记的 Ab2 α , 由于空间位阻的原因, Ab2 α 不能和 Ab2 β /

Ab1 复合物结合,而是识别捕获有待测物的那部分抗体的骨架区位点。最终读出的信号强度与抗体结合的待测物分子数量,也就是样品中待测物的含量成正相关。这一反应模式也被称为选择性抗体系统 (Selective Antibody System), Ab2 α 为选择抗体 (Selective Antibody)^[21]。基于抗独特型抗体的非竞争性免疫分析方法先后成功应用于雌二醇^[22]、皮质醇^[23]、甲氧基有机磷农药^[24]、玉米赤霉烯酮^[25]和呕吐毒素^[26]的测定。但是抗独特型抗体的制备有一定难度,阳性克隆率低,抗体分型筛选复杂,获得配对效果较好的 Ab2 α 和 Ab2 β 困难^[27]。

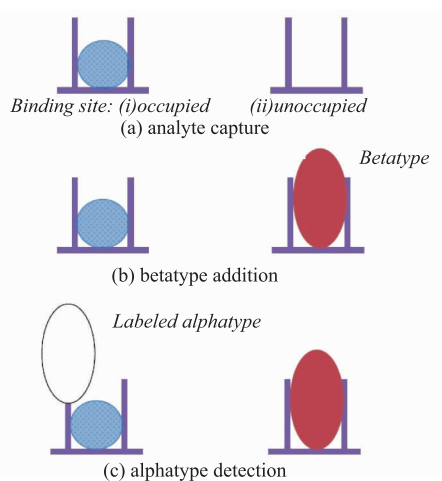


图 3 基于抗独特型抗体的免疫检测方法原理

Fig. 3 Principle diagram of anti-idiotype antibody immunoassay

4 基于抗免疫复合物抗体的非竞争性免疫分析方法

抗免疫复合物抗体是针对抗原与抗体结合后形成的新的抗原表位所产生的特异性抗体,又叫抗异型抗体 (Anti-metatype Antibody)。该抗体只能识别抗原抗体复合物,对单独存在的抗原或抗体几乎没有作用^[28]。据此,Self 等^[29]建立了基于抗免疫复合物抗体检测地高辛的非竞争性免疫分析方法。反应过程非常简单,酶标板上预包被有地高辛的特异性抗体 Ab1,后加入地高辛标准品或样品以及碱性磷酸酶标记的抗 Ab1/地高辛免疫复合物的抗体,室温孵育 10 min 后,显色读数。反应时间可缩短至 1 min,检测灵敏度并无明显降低。这种分析方法同样成功应用到血管紧张素 II 的测定^[30]。但是,这种抗免疫复合物抗体很难获得,主要原因在于抗体结合抗

原后引起的免疫复合物的空间构象变化细微,且半抗原高达 85%的可及表面包埋于抗体内部,难以形成新的有效的抗原决定簇。并且在筛选过程中,由于抗免疫复合物抗体与 Ab1 以及分析物组成的三元复合物相互间的接触表面积较大,无法实现对免疫复合物空间构象变化的精准识别,从而造成较高的非特异性干扰。基于噬菌体展示技术 (Phage-displayed Library)^[31-36] 和近年来发展起来的自主多样化库技术 (Autonomously Diversifying Library, ADLib)^[37-38] 则较好地解决了这一问题,在小分子化合物的非竞争性免疫分析领域有很好的应用前景。

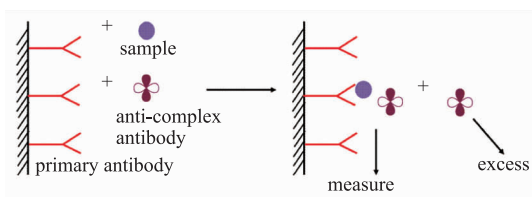


图 4 基于抗免疫复合物抗体的免疫检测模式

Fig. 4 Schematic diagram of anti-immune complex antibody based immunoassay

5 应用于半抗原检测的开放式夹心免疫分析法

1996 年 Ueda 等^[39]首次报道了在抗原存在时,抗体重链可变区 (VH) 与轻链可变区 (VL) 的结合稳定性得到增强这一现象,并提出了开放式夹心免疫分析法 (Open Sandwich Immunoassay, OS-IA) 的概念,见图 5。Suzuki 等^[40]通过表达抗 (4-羟基-3-硝基苯基) 乙酰基 (NP) 抗体的高亲和力突变体 VH 片段与大肠杆菌碱性磷酸酶的融合蛋白 (VH-PhoA) 以及 VL 片段与链球菌蛋白 G 的融合蛋白 (VL-PG) 构建了检测小分子化合物 NP 的开放式夹心免疫分析法。目前,开放式夹心免疫分析法已在玉米赤霉烯酮^[41]、赤霉醇^[42]、甲状腺素 T4^[43] 以及膝沟藻毒素^[44] 的测定上有成功的案例。然而,这种方法由于抗体的制备与筛选、编码 VH 与 VL 的 DNA 片段的构建以及融合蛋白的表达与纯化等过程较为复杂,而且并非所有的抗体 VH/VL 间的相互作用都与抗原有关,需要选择合适的抗体来建立 OS-IA 体系,在推广应用上仍存在一定的难度。

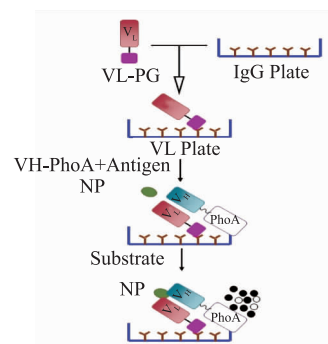


图 5 OS-IA 基本原理

Fig. 5 Schematic diagram of OS-IA

6 基于酶标记分析物-分析物抗体位点置换反应检测半抗原的非竞争性免疫分析方法

Giraudi 等^[45]提出了基于酶标记分析物-分析物抗体位点置换反应来检测半抗原的非竞争性免疫分析方法 (见图 6), 其反应过程大致如下: 固相包被抗体未被待测物占用的多余结合位点由封闭试剂封闭, 后由酶标记的分析物来全部置换结合了待测物的那部分抗体位点, 最终得到的信号强度与待测物结合的抗体位点数量成正比, 即是与待测物的含量成正比关系。这种分析方法后来又被应用到黄曲霉毒素的检测^[46]。这一反应体系的关键在于有效封闭试剂的制备。

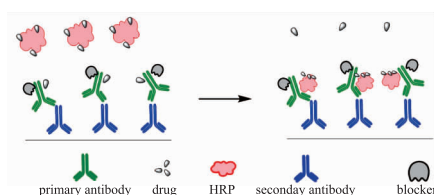


图 6 基于酶标记分析物-分析物抗体位点置换反应的非竞争性免疫分析方法的原理示意

Fig. 6 Schematic diagram of enzyme labeled analyte-analyte antibody site substitution reaction based non-competitive immunoassay

7 基于其他反应模式用于小分子化合物检测的非竞争性免疫分析方法

Piran 等^[47]以三碘甲状腺原氨酸 (T3) 为例, 建立了一种新颖的可检测单一表位半抗原的非竞争性免疫分析方法。它的反应过程大致如下: 吡啶酯

(AE) 标记的抗 T3 抗体与待测物在比色皿中孵育,形成 AE-抗 T3 抗体/T3 免疫复合物以及游离的 AE-抗 T3 抗体;于混合物中加入过量的表面固定有二碘甲状腺原氨酸(T2)的可控孔度玻璃珠(CPG),用以结合二价的 AE-抗 T3 抗体,而不能与上述免疫复合物作用;加入固化有抗 AE 抗体的顺磁性颗粒(PMP),捕获 AE-抗 T3 抗体/T3 复合物,而结合在 CPG 表面的 AE-抗 T3 抗体由于空间位阻作用不能被捕获。反应一段时间后,通过磁性分离检测 PMP 微粒表面的化学发光强度。

此外,基于亲和探针毛细管电泳技术(APCE)^[48-50]以及基于流动注射分析^[51-52]的非竞争性分析方法也广泛应用于小分子化合物的检测。

8 小分子化合物免疫分析检测技术展望

随着人类对食品和环境的质量日益重视,以及检测技术的不断进步,对农兽药、激素、食品添加剂以及有毒有害化学污染物等小分子物质的残留分析工作提出了更高的要求,残留检测技术正朝着快速、灵敏、多残留、高通量、低成本的方向发展。

相对于竞争法,非竞争法的优势有目共睹,发展空间广阔。但在应用非竞争免疫分析方法时,仍需强调以下几个问题或需要努力的方向:第一,在食品和环境监测领域的推广力度有待加大。当前,小分子物质非竞争免疫分析方法的应用多见于生物医药、临床诊断等生物医学分析领域,如对一些合成药物、生理活性物质的微量或超微量检测较为普遍,而在食品和环境中的残留或污染物的现场快速检测和控制领域涉及的相对较少。第二,对抗原抗体免疫识别机制及构效关系的系统而深入的基础理论研究有待加强。虽然免疫学分析技术发展较快,传统的抗体制备技术也已十分成熟,但获得高亲和力、灵敏度及特异性的抗体以及基于相应抗体建立切实可行方便的检测方法仍较为困难,其中一

个重要原因便是对分析技术背后的基础理论尤其是抗原抗体相互作用的结构基础及分子动力学过程的认识还不清楚、研究还不深入,在制备传统抗体时如何克服半抗原或抗原设计的随意性、盲目性,在筛选抗独特型或免疫复合物抗体时如何提高筛选效率、方便快捷地获得可与目标抗原精准识别的抗体分子及解决非特异性干扰,在建立开放式夹心免疫分析法时如何评估或测定 VH、VL 间的相互作用等等这些问题均需要相应基础理论的支持和指导。第三,与其他分离、富集和检测技术体系的联结有待加深。由于食品安全分析和环境评价对大批量样品快速筛查的需要,检测的高通量、自动化、标准化趋势愈发明显。如结合免疫亲和色谱技术、毛细管电泳技术可实现多目标分析物的快速分离和富集,结合流动注射分析技术可实现样品的连续自动分析,结合传感器、生物芯片等新型的信号放大系统可增强信号响应变化,提高检测灵敏度。上述系统在以非竞争法对食品和环境中的化学污染物残留进行现场检测过程中有着巨大的应用价值。

小分子化合物免疫分析技术的发展,除了需要方法学上的革新外,在核心试剂,如抗体或与抗体功能相似的生物识别材料,包括标记材料的改善上也应有所突破。比如说,常规的多克隆或单克隆抗体在目前的免疫分析领域仍具有不可替代的地位,但不可否认的是传统抗体在灵敏度、亲和力、稳定性以及识别多目标物的性能方面正遭遇着发展瓶颈。找寻一些新的生物识别材料,如受体蛋白^[53-55]、重组抗体^[56-57]、核酸适配体^[58-60]、分子印迹聚合物^[61-63]成为研究的热点。同时,一些新型的标记物,如镧系元素^[64-65]、量子点^[66-69]、纳米磁珠^[70-73]及核酸片段^[74-76]等的应用将极大促进免疫标记技术的发展,有望进一步提高免疫测定的灵敏度、特异性、稳定性和简便性。

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会 议 消 息

会议名称(中文): 首届中国生物工程学会青年工作委员会学术年会

所属学科: 生物技术与生物工程

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所在城市: 广东省 广州市

具体地点: 广州大学城

主办单位: 中国生物工程学会青年工作委员会

承办单位: 华南理工大学生物科学与工程学院

程序委员会主席: 逯光文、汪小我

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会议背景介绍: 中国生物工程学会青年工作委员会定于 2017 年 4 月 8-9 日在广州召开中国生物工程学会第二届青年科技论坛暨首届青年工作委员会学术年会 (China Society of Biotechnology Young Scientists Forum II)。本次论坛将延续首届论坛的传统, 程序委员会将从提交的会议摘要 (abstracts) 中选择青年科学家作专题报告; 同时将邀请国内外知名科学家作大会报告。本次论坛增加墙报交流环节 (poster session), 并评选中国生物工程学会青工委优秀墙报奖。