instead of a single one. DSM Andeno has used this method to resolve racemates on a scale of hundreds of kilograms.[10] Without a doubt "classical resolution" still has a great future, both on the fundamental and applied sides.

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In Vitro Evolution and Selection of Proteins: Ribosome Display for Larger Libraries**

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One of the most rapidly developing research areas since the beginning of the 90s is the generation and exploitation of molecular libraries (for a review see ref. [1]). The catchword is: combinatorial, paired with chemistry, biochemistry, or biology. Although the applied methods have hardly reached the text books, almost every researcher involved in the fields of chemistry and the biosciences is familiar with the term phage display, peptide, and nucleic acid libraries, or combinatorial chemistry. The common strategy as well as the goal of all chemical and biological approaches is always 1) to generate the largest and most comprehensive pool of molecules possible (the "library") and 2) to search through the library for individual substances with the desired properties such as, for example, for a starting substance (lead structure) for a drug or antibody for diagnostic purposes. Libraries are being used increasingly to identify interaction partners in signal transduction, cell – cell recognition, and immunological control processes. These kinds of approaches accelerate not only the handling of many problems, but also often provide the first possible means to solving them. Therefore, numerous chemists and biologists in industry and university labs have begun to develop novel methods that make it possible to create, analyze, and propagate libraries. The best-know procedure amongst the many described[1] is the so-called phage display method,[2, 3] where the peptide or protein library is expressed on the phage surface. The selection of the desired phage is achieved in vitro, whereas the amplification of the phages takes place in bacteria, that is, in vivo. Here lies a decisive problem with the use of this biologically generated repertoire. The size of the library, is limited by the first essential step of introducing the phage DNA, which contains the genes that code for the individual components of the library, into cells. This step is known as transfection or transformation and permits a repertoire size of between 10^7 and 10^9 different components.[4] In addition, the environment of the host cell imposes an additional selection pressure that may work against the desired variant.[5] A further "disadvantage" of phage display is the time consuming shuttling between in vitro and in vivo steps.

These problems could be avoided if the isolated protein synthesis machinery of the cell (ribosome) was used instead of intact cells for the production of peptide and protein libraries. This was successful first of all in the search for peptides with the desired properties by using the so-called polysome display.[6, 7] A polysome is a complex comprised of one mRNA and a number of ribosomes. A breakthrough was achieved by Hanes and Plückthun[8] who worked out a system for the general application of in vitro selection and evolution of proteins, which they called ribosome display. Selection and evolution systems are based on the following criteria: 1) generation of molecular diversity, 2) selection of molecules with

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desired properties, 3) amplification of the selected molecule, and 4) repetition of steps 2 and 3, or analysis of the selected molecule. If mutations are permitted during the amplification procedure, the system possesses an evolutionary character. The addition of nucleic acids facilitates problem-free, and very effective amplification of the required information. In step 2 it is possible to translate the information from the nucleic acids (genotype) into a protein, which can then be selected for according to its function (phenotype). The genotype and phenotype must be linked to each other and selected together so that the information (genotype) after selection can be amplified. Usually for such systems comprised of associated genotype and phenotype, such as phage display, time-consuming alternate in vitro and in vivo steps are required. However, the combination of genotype and phenotype with ribosome display functions without the aid of cells. Proteins are synthesized in vitro based on the coding by a mRNA, after which each mRNA molecule together with the translated protein remains attached to the ribosome. This complex can then be selected according to the phenotype of the protein.

The method functions according to the following principle (Figure 1): a DNA library encoding the desired protein is created. By using the polymerase chain reaction (PCR, for a review see ref. [9]) some regulatory elements, as clarified later, are attached to the DNA. By in vitro transcription the DNA is copied into a mRNA, which is translated in a prokaryotic in vitro system,[10] which gives rise to the ribosomal complex comprised of mRNA, ribosome, and translated protein. Various optimizing steps sustain the stability of the complex and permit correct folding of the protein. The complex is selected on the basis of the desired binding properties of the translated protein. During elution the complex dissociates and the mRNA is released. It is then isolated, converted by reverse transcriptase into cDNA (complementary DNA), and amplified by PCR. The resulting DNA can be introduced into the next selection cycle or analyzed by cloning and sequencing.

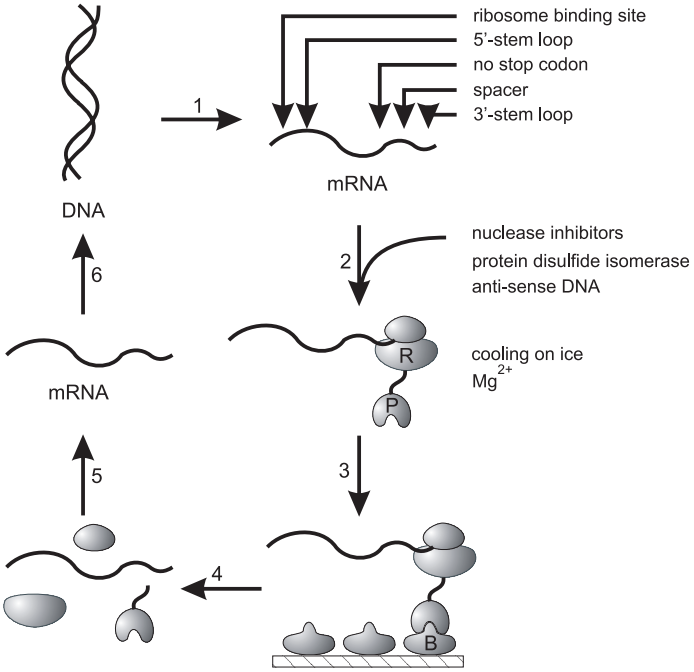

Figure 1. The principle of ribosome display. Step 1: a DNA library is amplified by PCR, which at the same time introduces the structural elements indicated, and then transcribed into RNA. Step 2: the mRNA is converted (translated) into a protein in an in vitro system. The addition of protein disulfide isomerase, nuclease inhibitors, and anti-sense DNA improves protein folding and protects the mRNA and protein. Step 3: the complex of ribosome (R), mRNA and protein (P) with the desired binding property is stabilized by cooling and addition of Mg^{2+}, and is selected with regard to its affinity for an immobilized binding partner (B). Unbound complexes are removed by washing. Step 4: elution of the bound ribosome complex. Step 5: isolation of the mRNA. Step 6: reverse transcription of RNA into DNA and amplification by PCR. The DNA is subjected to a further round of enrichment or can be analyzed by cloning and sequencing. Usually five cycles comprising these steps are carried out.

The ribosome display method was developed by using a single chain antibody (scFv) fragment as a model system and optimized and developed by numerous additions into a system for general application (Figure 1). These improvements protect the mRNA from enzymatic breakdown by nucleases, stabilize the ribosome complex, and facilitate the correct folding of the presented protein, and therefore increases significantly the overall efficiency of the method. As a measure of efficiency, the yields of the eluted mRNA that is isolated after the affinity selection with the antibody's binding partner (antigen), in this case a peptide, are compared. Here, the structure of the mRNA was found to play a significant role. A hairpin loop is introduced at both the 5′ and 3′ ends to protect the mRNA from exonucleases. The hairpin loop at the 3′ end is also responsible for the termination of translation since the mRNA contains no stop codon. If translation was to end at a stop codon this would lead to dissociation of the ribosome complex, which would then no longer be available for affinity selection. A spacer is introduced between the coding sequence for the antibody and the 3′-hairpin loop. This ensures that the actual polypeptide chain has already completely migrated out of the ribosome when transcription stops, and can fold up unhindered. Efficiency is further increased by the addition of nuclease inhibitors and protein disulfide isomerase, which catalyzes the formation of the correct disulfide bridges of scFv. A further, particularly noticeable, improvement relates to the inhibition of a protein degradation pathway: recently a peptide marking system was reported where proteins that derive from a mRNA without a stop codon are marked for degradation with a peptide coded for by a special RNA.[11] Indeed, through blocking of this RNA by the corresponding complementary (anti-sense) DNA the yields could be increased further.

Through the described structural changes in the mRNA and the reagents, which are added during the translation, the total yields were increased from 0.001 % to 0.2 %. Subsequently, a test experiment was carried our to assess how well a protein with a desired function can be enriched from a mixture of two proteins. Two mRNA constructs were created that were identical in the properties described above, one coding for an anti-hemagglutinin scFv[12] and the other for an anti-β-lactamase scFv.[13] These were mixed in a ratio of $1:10^8$ and selected in five cycles (Figure 1) on immobilized hemagglutinin peptide. At the end of the selection 90 % of the ribosome complexes contained the anti-hemagglutinin antibody fragment. Per cycle, therefore, the desired protein was enriched

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100-fold. After the fifth round 20 clones were sequenced and of these 18 coded for the intended protein. Table 1 shows the clones arranged according to their relative binding strength and lists the mutations found. Two clones corresponded to the wild-type sequence, the rest showed one to four amino acid exchanges, which demonstrates the range of possible mutations that retained or even improved upon the original function.

Table 1. Mutations in the selected anti-hemagglutinin single chain antibody.[a]

Clone no.	Mutation V_L	V_H	linker	Binding strength
12	K45R	P41R		102
10		S30P		101
6				100
2				96
7		G16D		89
3	Y49H			86
9	E55G, E105G	A23V	S5P	86
1	T20A		G12E	80
18	N(27d)D	D10G, T50N		76
4	V13A, K28R	V12A		72
13	V58I	Y79C		65
16	L83R	I51V, K75E, A113V		63
14	K30E	D61G	S5P	43
17	F71S			18
8	E17G, K18E, K30E			14
5	L11P		G13D	9
11	S(27e)F, N90E	V48I		9
15	G64D, S77A	G15S		6

[a] For all the analyzed antibody clones the relative binding strength and the mutation(s) found in the three regions of the antibody (V_L = variable region of the light chain; V_H = variable region of the heavy chain; and the linker that connects both regions. The mutations are numbered according to Kabat et al.[18] Owing to the variable length of the complementary binding regions (CDRs), several positions are additionally labeled with lowercase letters.

This experiment from Hanes and Plückthun showed that ribosome display is suitable as a purely in vitro system for phenotypic selection following ligand binding. Also the correct folding and formation of disulfide bridges was feasible, which for a large proportion of proteins is essential. Therefore, ribosome display represents the first complete system that exists in vitro for the evolution and selection of folded native proteins. The transformation of DNA into cells is not required, and nothing more stands in the way of synthesizing and screening large protein libraries ($\gg 10^9$ single components). He and Taussig[14] recently succeeded in performing reverse transcription directly within the ribosomal complex with an analogous eukaryotic transcription/translation system, without previously purifying the mRNA. This could mean reduction in the time necessary for one cycle of ribosome display to 8 h. In addition, new methods such as "sexual PCR",[15] error-prone PCR,[16] or PCR with trinucleotide primers[17] could further increase the diversity of ribosome display. On the other hand, the error rate in ribosome display could be reduced by using polymerases with error correction or proofreading activity, if the method is to be used merely for searching through libraries.

Ribosome display has great potential to advance basic research in the areas of protein structure, folding, and evolution. At the same time the method greatly accelerates the process of searching for new lead compounds with interesting properties, as well as optimizing already known therapeutically relevant proteins. Furthermore, valuable insights into the exact location of active sites on protein surfaces may be gained, which will simplify the design of protein mimics and a new class of therapeutic substances.[3]

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